Novel insights into MACC1 transcriptional regulation for identifying small molecule MACC1 inhibitors to restrict colorectal cancer progression

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Prof. Dr. Jan-Hendrik Olbertz

Dekan der Mathematisch-Naturwissenschaftlichen Fakultät I

Prof. Stefan Hecht, Ph.D.

Gutachter: 1. Prof. Dr. Ulrike Stein

2. Prof. Dr. Achim leutz

3. PD Dr. med. Thorsten Cramer

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This study was conducted at the Max Delbrück Centrum for Molecular Medicine Berlin-Buch in the research group of Prof. Dr. Ulrike Stein.

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ABBREVIATIONS

ACF	Aberrant crypt foci
APC	Adenomatous polyposis coli
AP-1	Activating protein-1
ARNSHI	Autosomal recessive non-syndromic hearing impairment
BMP	Bone morphogenetic protein
BAX	Bcl-2-associated X protein
Bcl2	B-cell lymphoma 2
CD44	Cluster of differentiation 44
CIN	Chromosomal instability
C/EBP	CCAAT-enhancer-binding proteins
CIMP	CpG island methylator phenotype
CMV	Cytomegalovirus
CRC	Colorectal cancer
CTNNB1	β-catenin
CT	Computed tomography
DCC	Deleted in Colorectal Cancer
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ECM	Extracellular matrix
EMT	Epithelial mesenchymal transition
ERK	Extracellular-signal-regulated kinases
5-FU	Fluorouracil
FAP	Familial adenomatous polyposis
FDA	Food and Drug Administration
FFS	Failure-free survival
G418	Geneticin
GFP	Green fluorescent protein
GIPC1	GIPC PDZ domain containing family, member 1
GSK-3β	Glycogen synthase kinase -3β
HGF	Hepatocyte growth factor
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HNPCC	Hereditary nonpolyposis colorectal cancer
HTS	High throughput screening
IL-4	Interleukin 4
IFN-γ	Interferon-γ
IGF2R	Insulin-like growth factor 2 receptor
iPSC	Induced pluripotent stem cell
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LEF-1	Lymphocyte enhancer factor-1
LPS	Lipopolysaccharides
Lgr5	Leucine-rich repeat-containing G-protein coupled receptor 5
LOH	Loss of heterozygosity
MACC1	Metastasis associated in colon cancer 1
mAb	Monoclonal antibodies
MAPK	Mitogen-activated protein kinase
MLH1	MutL homolog 1
MMR	Mismatch repair
MMP	Matrix metalloproteinase

MSI	Microsatellite instability
OS	Over-all survival
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol-3 kinase
RFS	Relapse-free survival
RTK	Receptor tyrosine kinases
SASH1	SH3 domain-containing protein 1
SNP	Single-nucleotide polymorphism
SCID	Severe combined immunodeficiency
Sp1	Specificity protein 1
SPECT	Single-photon emission computed tomography
TBP	TATA-binding protein (TBP)
TAF	TBP-associated factor
TCF	T-cell factor
TGFRII	TGF-β receptor 2
TGF-β	Tumor growth factor-β
TIAM	The invasion-inducing T-lymphoma invasion and metastasis 1
TP53	Tumor protein p53
TLR4	Toll-like receptor 4
TSS	Transcription start site
UTR	Untranslated region
WISP3	WNT1-inducible-signaling pathway protein 3
Wnt	Int1 and wingless (drosophila homolog)
ZEB1	Zinc finger E-box-binding homeobox 1

ABSTRACT

Metastasis-mediated mortality remains a major challenge in the management of colorectal cancer (CRC). One of the key findings in understanding molecular pathogenesis of colorectal cancer metastasis is the identification of the new gene MACC1. MACC1 has been reported as a prognostic biomarker for tumor progression and metastasis-free survival in CRC along with other solid tumors. The five-year survival rate has been shown to be 80% for subjects with low MACC1 mRNA expression and only 15% for subjects with high MACC1 expression. It also induces cell motility and proliferation in cell culture and metastasis in mouse models. Consequently, targeting MACC1 to intervene in tumor progression and metastasis formation holds a promising approach to treat CRC patients.

We designed a strategy to inhibit MACC1 via targeting its transcription. Therefore, we started with the identification of the MACC1 gene promoter by creating various promoter-luciferase constructs and studied its transcriptional regulation machinery. Using site directed mutagenesis, chromatin immunoprecipitation and electrophoretic mobility shift assays, we established that transcription factors such as Ap-1, Sp1, C/EBPs and GIPC1 bind to the MACC1 promoter and govern the transcription of the MACC1 gene. RNAi technology followed by analysis of a panel of colorectal carcinomas of various stages established the role of these transcription factors in MACC1-induced cell motility *in vitro* and *in vivo*.

Further, by employing high throughput screening targeting the MACC1 promoter, we identified the very first small molecule MACC1 inhibitors, Rottlerin and Lovastatin. Rottlerin and Lovastatin were shown to specifically act on the endogenous MACC1 promoter leading to reduced MACC1 expression in a time- and concentration-dependent manner. Further *in vitro* functional assays demonstrated the impact of the small molecule inhibitors on retarding cell proliferation and motility. Both the small molecule inhibitors restricted Sp1 levels and interfered with the binding of c-Jun to the MACC1 promoter, thereby inhibiting MACC1 transcription and MACC1-induced proliferation and migration. The study further described the effect of Rottlerin on a CRC-xenografted mouse model. Daily treatment of xenografted mice with Rottlerin resulted in the inhibition of MACC1 expression in the primary tumor accompanied with the restricted tumor growth.

To summarize, this is the first study unraveling the MACC1 promoter and its transcriptional regulation. This knowledge was then implicated to identify small molecules, Rottlerin and Lovastatin as newly identified MACC1 inhibitors. In clinical settings, inhibition of MACC1 expression using these inhibitors might provide immense potential for the treatment of CRC patients who are at high risk for MACC1-induced metastasis linked to shorter survival.

ZUSAMMENFASSUNG

Die Metastasen-bedingte Mortalität bleibt weiterhin eine große Herausforderung in der Behandlung des kolorektalen Karzinoms (KRK). Einen wesentlichen Fortschritt zum Verständnis der molekularen Pathogenese des Metastasierungsprozesses im KRK trug die Identifizierung des neuen Gens MACC1 bei. MACC1 wurde als prognostischer Biomarker für die Tumorprogression und das Metastasen-freie Überleben im KRK sowie in anderen soliden Tumoren beschrieben. Die 5-Jahres-Überlebensrate betrug hierbei 80% für Patienten mit niedriger MACC1 mRNA Expression im Vergleich zu nur 15% für Patienten mit hoher MACC1 Expression. Das Gen induziert Zellmotilität und Proliferation in Zellkultur sowie die Metastasierung im Mausmodell. Letztendlich stellt MACC1 ein vielversprechendes Ziel für die Intervention bei Tumorprogression und –metastasierung und damit für die Behandlung von KRK-Patienten dar.

Unser Versuchsvorhaben war es, MACC1 über seine transkriptionelle Expression zu inhibieren. Hierfür identifizierten wir zunächst die Genpromoter-Region von MACC1 durch das Erstellen verschiedener Promoter-Luciferase Konstrukte und untersuchten MACC1s transkriptionelles Regulationsnetzwerk. Durch ortsgerichtete Mutagenese, Chromatin Immunopräzipitation und Electrophoretic Mobility Shift Assay ermittelten wir, dass Transkriptionsfaktoren wie Ap-1, Sp1, C/EBPs und GIPC1 an den MACC1-Promoter binden und die Transkription des MACC1-Gens kontrollieren. Durch Verwendung von RNAi-Technologie und der Analyse einer KRK-Patientenkohorte mit verschiedenen Tumorstadien etablierten wir die Rolle dieser Transkriptionsfaktoren in der MACC1-induzierten Zellmotilität *in vitro* und *in vivo*.

Darüberhinaus konnten wir durch Hochdurchsatz-Screening die bisher ersten Kleinmolekül-Inhibitoren gegen MACC1 identifizieren: Rottlerin und Lovastatin. Wir zeigten, dass Rottlerin und Lovastatin spezifisch auf den endogenen MACC1-Promoter wirkten, was eine zeit- und konzentrationsabhängige Reduktion der MACC1-Expression zur Folge hatte. Weitere funktionelle Versuche *in vitro* demonstrierten, dass die Kleinmolekül-Inhibitoren ausschlaggebend für die Verminderung von Zellproliferation und -motilität waren. Beide Inhibitoren begrenzten das Expressionsniveau von Sp1 und interferierten mit der Bindung von c-Jun mit dem MACC1-Promoter, was in einer Inhibition der MACC1-Transkription sowie der MACC1-induzierten Proliferation und Migration resultierte. Ferner führte die tägliche Behandlung von Xenograft-Mausmodellen mit Rottlerin zu einer Inhibition der MACC1-Expression im Primärtumor und einer damit einhergehenden Begrenzung des Tumorwachstums.

Zusammenfassend lässt sich festhalten, dass in der vorliegenden Arbeit zum ersten Mal der MACC1-Promoter und seine transkriptionelle Regulation beleuchtet wurden. Die neuen Erkenntnisse wurden außerdem zur Identifizierung der ersten Kleinmolekül-Inhibitoren gegen MACC1 genutzt. Zur Behandlung von KRK-Patienten mit einem hohen Risiko für MACC1-induzierte Metastasierung und damit kürzerer Überlebenszeit, könnten diese Inhibitoren enormes Potential für die klinische Anwendung beherbergen.

1. INTRODUCTION

1.1 Colorectal cancer: Incidence and epidemiology

Colorectal cancer (CRC) is the third most common cancer in men and second in women, worldwide [1]. CRC dominates over all gastrointestinal cancers and continues to be in the spotlight owing to its high prevalence and long progression time of the premalignant lesion along with expensive and invasive screening procedures. The highest rate of incidence among both genders are reported in east European countries (Czech Republic and Slovakia), Japan (Miyagi), New Zealand, Australia, Germany, and among African Americans, whereas the lowest rates are found in Africa, Central and South America, and South Central Asia (India and Pakistan) [1]. Five-year survival rates for CRC in developing countries range from 28% to 42% [2, 3], compared to more than 60% in the United States, Japan, and Switzerland [4, 5]. Within Germany, CRC is the second most common cancer and third most common cause of cancer associated mortality in female whereas in male, it is the third most common cancer and second most common cause of cancer associated mortality [6]. The overall age adjusted survival rate for CRC patients within Germany over the period 2002-2006, has significantly increased from 60.6% to 65%. This increase is specific for patients with localized cancer as no improvement in overall survival has been achieved for patients with metastatic disease [7]. A multitude of risk factors have been linked to CRC, majority being sporadic. Risk factors include increasing age, diet, lifestyle (eg. high consumption of red meat, fat-rich diet, lack of exercise and obesity, inadequate fiber intake, excessive smoking and alcohol), environmental exposures, and chronic inflammatory syndromes affecting the gastrointestinal tract (Ulcerative colitis and Crohn's disease) [8]. Inherited conditions such as familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC), also called Lynch syndrome, account for approximately 5 to 10% of all CRC cases [9]. HNPCC is associated with mutations in genes involved in the DNA repair pathway, namely the MLH1 (MutL homolog 1, colon cancer, nonpolyposis type 2) and MSH2 (MutS protein homolog 2) genes [10] and FAP is caused by mutations in the tumor suppressor gene APC [11]. They will be discussed in detail in the later sections.

1.2 Tumorogenesis and cancer progression

Tumorogenesis of CRC is a complex process involving both environmental and genetic factors [12]. This multistep process is an outcome of the interactions between environmental influences, germ-line factors dictating individual cancer susceptibility and accumulated somatic changes in colorectal epithelium [13]. Several concepts have been established to

understand the molecular origin of CRC. Researchers, worldwide are still trying to pinpoint the exact mechanism that initiate tumor development, its progression into an invasive and metastatic phenotype and its responsiveness or resistance to therapies. Colorectal carcinogenesis involves two major pathways. One of them is called the "*canonical*" (adenoma-carcinoma sequence) or "*suppressor*" or "*traditional*" pathway and involves chromosomal instability (CIN) leading to tumor progression [14]. This traditional pathway is characterized by mutation in the APC gene or allelic losses on chromosome 5q (APC gene), mutation in KRAS, loss of 18q (DCC/SMAD4) and 17p (p53).

The second pathway of colorectal carcinogenesis involves microsatellite instability (MSI), and is called as the "*mutator*" pathway. It is characterized by defects in the mismatch repair system (MMR genes) thereby interruption of the DNA damage repair system (Fig. 1.1) This pathway accounts for approximately 15%-20% of sporadic CRCs [15]. These two pathways present different molecular and clinical behavior along with distinct histopathological features and are described in the next section.

1.3 Canonical pathway

This pathway is responsible for the development of a majority of colorectal carcinomas and is based on the Fearon and Vogelstein model of colorectal carcinogenesis. Fearon and Vogelstein proposed specific genetic events with evolving tissue morphology (Figure 1.1) leading to CRC progression. Alterations in tumor suppressor genes (APC, p53, DCC) and oncogenes (KRAS, CTNNB1 encoding β-catenin) with high frequency of allelic imbalance, chromosomal amplification and translocations are characteristics of this pathway. These will be discussed in details along with the signaling pathways they affect in the following sections.

1.3.1 APC, β-Catenin and Wnt signaling

The adenomatous polyposis coli (APC, 5q21) gene is mutated in approximately 60% of cancers arising from the colon and in 82% of cancers in the rectum [16]. It is a major unit of a multi-protein (APC-axin-GSK3β) complex in the Wnt pathway.

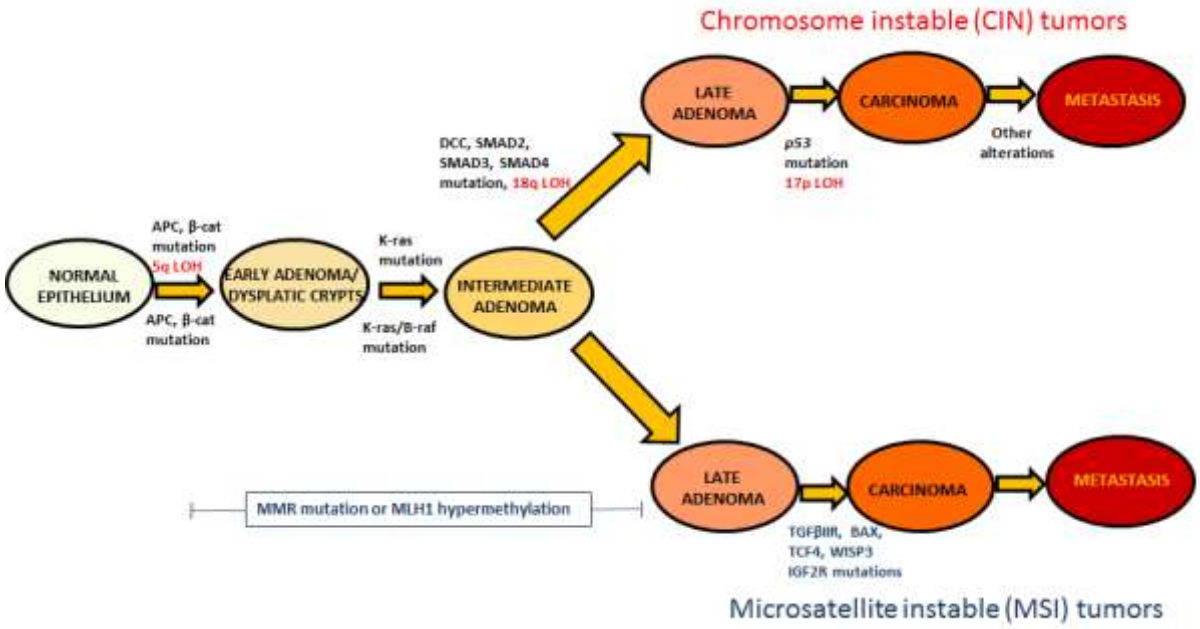


Figure 1.1: Two major pathways for CRC. CRC progression follows a series of genetic and epigenetic changes giving rise to adenoma-carcinoma sequence. The majority of CRCs show chromosomal instability and are characterized by gain or loss of chromosome 5q (APC), 18q (DCC, SMAD4) and 17p (p53) as shown in red, whereas a subgroup of CRCs show defects (methylation of promoter or mutations) in DNA mismatch repair genes (in blue) leading to accumulation of mutations in microsatellite sequences. Modified after Moran et al. [17, 18]

This multi-protein complex binds to β-catenin and causes its phosphorylation, subsequent ubiquitination, and thus ultimately leading to its destruction in the proteasome. APC mutations interfere with APC-β-catenin binding, impairing the degradation of cytoplasmic β-catenin, which is then available to translocate to the nucleus resulting in constitutively active Wnt-signaling pathway. However, in tumors lacking APC mutations, mutations at codon 31, 33, 37 and 45 of exon 3 of the CTNNB1 gene render the protein resistant to APC-degradation and act as an alternate way of activating the Wnt signaling [19]. The frequency of APC or β-catenin mutations in early adenomas is as high as 80 to 85% suggesting that these genetic alterations initiate tumorogenesis in the intestine in a process characterized by the formation of dysplastic crypts and early adenomas [20, 21].

In addition, APC also plays a role in regulation of mitosis by ensuring correct chromosomal alignment and subsequent segregation during metaphase [22]. Therefore, loss of APC results in chromosomal abnormalities during metaphase contributing to CIN [23]. Hence, mutations in APC and the Wnt-pathway are a frequent mechanism for progression of early lesions to more advanced stages of CRCs. However other genetic alterations may also serve as additional molecular surrogates deciding the severity of colorectal carcinogenesis.

1.3.2 KRAS and MAPK signaling

The KRAS proto-oncogene is a GTP-binding protein located at chromosome 12p12.1. It regulates several effector pathways involved in cell proliferation, survival and metastasis [24]. GTP bound KRAS (active Ras protein) phosphorylates MAP3K (Raf), which further activates MAP2K (MEK-1/2), subsequently phosphorylating MAPK (ERK-1/2), thus activating the MAPK signaling cascade. Although MAPK cascade is also activated by other non-KRAS-mediated growth factor pathways, signaling in cancer is frequently deregulated through activating mutations in KRAS or BRAF [25].

Oncogenic KRAS mutations are detected in approximately 40% CRCs with most frequent mutations in codon 12 and codon 13 [26]. These mutations are more frequent in advanced adenomas despite being frequent up to 63% in sporadic dysplastic aberrant crypt foci (ACF). Thus, KRAS is not sufficient to drive carcinogenesis but certainly confers a growth advantage for the progression of CRC [27]. KRAS mutations are present in both adenomas and hyperplastic polyps suggesting that KRAS mutations are not the predominant precursors of either type of colorectal tumor in contrast to APC mutation which is closely associated with the initiation of adenomas [21].

1.3.3 SMADs, DCC and TGF-β signaling

DCC (deleted in colorectal cancer), SMAD2 and SMAD4 genes are all located at chromosome 18q21.1. Approximately 60% of CRCs show allelic loss at this site [28]. Germ line mutations in SMAD4 can cause juvenile polyposis syndrome, associated with gastrointestinal polyps and cancer [29]. In sporadic CRCs, so far it was known that SMAD4 mutations are more influential in colorectal carcinogenesis and are often reported in advanced stages of CRCs [30, 31]. However, a recent study demonstrates first substantial contribution of SMAD2 and SMAD3 in sporadic CRCs occurring in 3.4% and 4.3% of primary cancers, respectively [32]. SMADs are TGF-β superfamily members and have been shown to control proliferation, differentiation, migration and apoptosis of many different cell types [33]. TGF-β is an important inhibitor of growth [34]. In colorectal carcinogenesis, an escape from TGF-β induced-inhibition of proliferation has been observed leading to uncontrolled growth [35]. SMADs play a central role in TGF-β signaling. They are divided into three groups based on their role in signal transduction. First group comprises of receptor activated SMADs wherein, SMAD2 and SMAD3 are phosphorylated by different type I serine threonine receptors of the TGF family. SMAD1, SMAD5 and SMAD8 are phosphorylated by receptors for bone morphogenetic proteins (BMPs). Second group consists of common mediator SMAD4, which forms complexes with receptor-activated SMADs and translocate these

complexes to the nucleus for regulating target gene expression. The third SMADs category is inhibiting SMADs, SMAD6 and SMAD7. These inhibitor SMADs either block phosphorylation of receptor-activating SMADs or compete with complex formation with SMAD4 [36]. Activated SMADs regulate diverse biological effects by cooperating with transcription factors resulting in cell-state specific modulation of transcription.

DCC encodes a transmembrane receptor which binds to its ligand, netrin-1. Wild-type DCC in the absence of netrin-1, promotes apoptosis by activating caspase-3 as well as induces G2/M cell-cycle arrest in some cell lines [37]. Also, in the presence of netrin-1, DCC activates Rac-1 which contributes towards actin organization and cell motility [38].

1.3.4 TP53

Tumor suppressor gene TP53 encoding p53 is located on chromosome 17p13.1. The functional p53 gene facilitates DNA repair by inducing G1 cell-cycle arrest and by elevating the expression of cell-cycle retarding genes thereby providing sufficient time for DNA repair during environmental or oncogenic stress [39, 40]. In case of extreme genetic stress, it induces apoptosis which is a major p53 associated cellular process [41]. Mutation or loss of heterozygosity (LOH) in this gene is associated with various human cancers [42]. Particularly, in CRC's, as described by Fearon and Vogelstein adenoma-carcinoma sequence, p53 associated abnormalities are late events. It marks the transition from pre-invasive adenoma to invasive cancer disease. The frequency of p53 alterations (4%-26% of adenomas, 50% of adenomas with invasive foci, and in 50%-75% of CRCs) increases with the progression of lesion within the pathway [14, 43]. Thus to conclude, p53 gene usually remains unaffected throughout the progression from normal mucosa to adenoma development. During a later event of tumorogenesis, however, expression of wild-type p53 may become rate limiting for cell growth. At this point, p53 point mutations and allelic deletions might occur very rapidly and are likely to contribute to progression to carcinoma stage [44].

1.4 Microsatellite instablilty pathway

The majority of CRCs follow traditional pathway involving chromosome instability characterized by loss or gain of chromosome arms, translocations, or gene amplifications. But nearly 15%-20% of sporadic CRCs display ‘‘mutator’’ phenotype or Microsatellite instablilty pathway (MSI) pathway [45]. In contrast to CIN tumors, they are characterized by inactivation or mutation in DNA mismatch repair (MMR) genes. The MMR system consists of at least 7 genes hMLH1, hMLH3, hMSH2, hMSH3, hMSH6, hPMS1 and hPMS2 which

associate with their partners MutS and a MutL, to form functional heterodimers [46]. Defects in the MMR system leads to accumulation of errors in DNA, mainly frame shift mutations, within short repeat sequences referred to as Microsatellites (MSI) [47]. Mutations or epigenetic changes in hMLH (often silencing caused by methylation) and hMSH2 are key components of MMR system and thus the most common cause of MSI-H in sporadic CRC and in HNPCC, Hereditary Non Polyposis Colorectal Cancer [15, 46]. One of the consequences of germ line MMR mutation is Lynch syndrome, responsible for one fifth of all MSI CRCs. The more common non-familial MSI CRC is due to epigenetic inactivation of MLH1 along with the hypermethylation of the surrounding promoters of multiple genes, i.e. CpG island methylator phenotype (CIMP+) [48, 49]. These sporadic tumors also show co-occurrence of BRAFV600E mutations that serve to distinguish them from Lynch syndrome [50]. The most intriguing difference between CIN and MSI tumors is that the former shows one of the hallmark of cancer i.e. aneuploidy whereas MSI-H cancers are usually not aneuploidy [51]. Instead in MSI tumors, mutations described in microsatellite sequences are present in genes such as TGFβRII, BAX, TCF-4, WISP3, IGF2R [52-56]. Additionally MSI-H tumors have distinct clinical and histopathological features such as proximal colon predominance, poor differentiation and/or mucinous histology and low frequency of distant metastases. The prevalence of MSI is more common among stage II compared with lymph node–positive or stage III CRC. MSI is relatively uncommon among stage IV or metastatic CRC (4%) [57]. Thus, it is important to identify the right carcinogenic pathway in patients. Therefore, MSI testing should be incorporated on several different sporadic polyps, including hyperplastic polyps, tubular adenomas, serrated adenomas and mixed polyps to investigate whether the adenoma is the precursor lesion in the mutator or MSI pathway, in order to design effective treatment strategies.

1.5 Epithelial-Mesenchymal transition and CRC metastasis

90% and 75% of patients in stage I and stage II respectively of their disease are cured effectively via surgical resections. However, only 15% of the patients with advanced CRC survive for more than 5 years. One of the hallmarks of malignant transformation is the ability of tumor cells to invade and metastasize [58]. This metastatic dissemination of primary tumors to localized or distant organs bears life- threatening consequences. Approximately 90% of all cancer deaths arise from metastatic growths, leading to compromised treatments and a worse disease outcome [59]. Epithelial-Mesenchymal transition (EMT) is the switch that facilitates the tumor to acquire an invasive and aggressive phenotype.

EMT is a reversible process in which epithelial cells lose their cell-cell adhesion systems, their polarity and gain mesenchymal properties with increased migratory capabilities (Fig. 1.2). Cancer cells via EMT pathway detach from the neoplasm, enter into subsequent steps of the invasion-metastasis cascade, migrate, enter into blood or lymphatic vessels and disseminate into various organs to cause distant metastases [60].

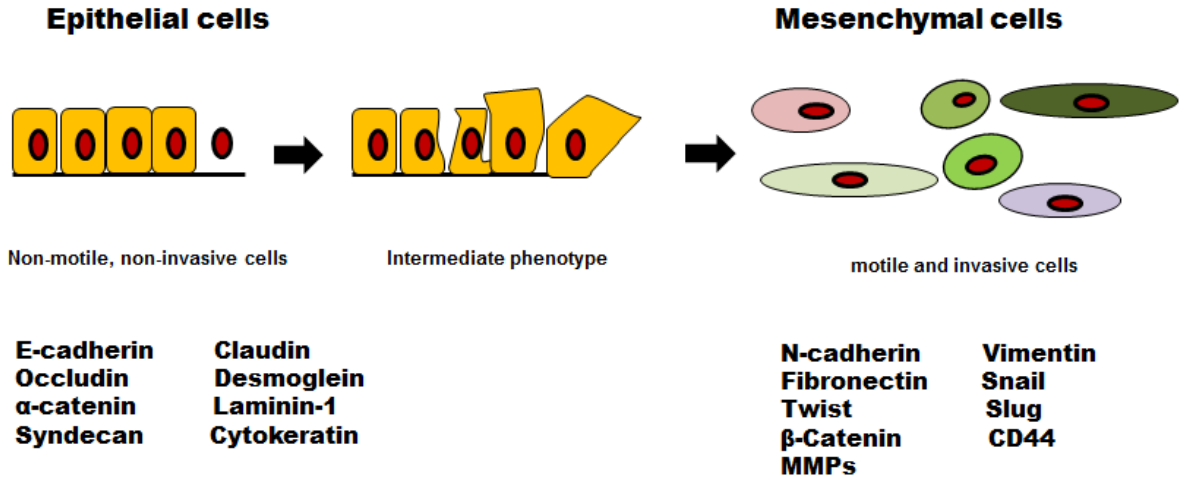


Figure 1.2: The epithelial-mesenchymal transition in cancer invasion and metastasis. EMT is a morphogenetic process in which epithelial cells lose their properties and acquire mesenchymal characteristics during embryogenesis and cancer progression. Various mesenchymal cells can be derived from primary epithelial cells. Modified after Kalluri et al. and Gout et al. [61, 62]

The nature and type of signaling mediators that contribute to EMT of cancer cells remain unknown. One of the hypothesis states that the tumor associated stromal factors like Hepatocyte growth factor (HGF), Epidermal growth factor (EGF), Platelet- derived growth factor (PDGF), and TGF-β induce EMT and activate EMT-inducing transcription factors, notably Snail, Slug, zinc finger E-box binding homeobox 1 (ZEB1), Twist, Goosecoid, and FOXC2 [61]. However, a series of signaling pathways involving Extracellular signal-regulated kinases (ERK), Mitogen-activated protein kinases (MAPK), Phosphatidylinositol-3-kinase (PI3K) , Akt, SMADs, RhoB, β-catenin, lymphoid enhancer binding factor (LEF), RAS, and C-FOS as well as cell surface proteins such as β4 integrins, α5β1 integrin, and αVβ6 integrin also govern EMT [63]. One of the well-established and major events of EMT is the loss of functional E-cadherin involved in cell-cell adhesion [64]. The extracellular domain of trans-membrane E-cadherin interacts with the E-cadherin expressed on adjacent cells, and the intracellular domain binds to β-catenin. This β-catenin along with other proteins binds to the cytoskeleton. All these together maintain cell–cell adhesion, cell shape, polarity, and cell migration [65].

Particularly in CRC's, loss of functional E-cadherin is linked with peripheral accumulation of Src and phospho-myosin, increased guanine nucleotide exchange factor TIAM (The

invasion-inducing T-lymphoma invasion and metastasis 1) and an elevated RhoC expression and up-regulation of Snail [66-69]. In addition EMT is associated with a series of molecular alterations which include a decrease in expression of epithelial markers and gain in expression of mesenchymal markers (Fig. 1.3) [62]. It is also proposed that EMT imparts the cancer cells not just migratory abilities but also self-renewal capability by inducing stem cell state [70]. Consistent to this, Snail induced EMT also confers a stem cell like behavior as shown by the overexpression of stem cell markers like CD133 and CD44 in CRCs [69].

Finally, the disseminated cells after reaching a particular distant site shed their mesenchymal characteristics by the process of mesenchymal-epithelial transition (MET) for secondary tumor formation and proliferate again for building up metastases. Thus EMT is the central mechanism for progression of carcinomas into the metastatic stage (Fig.1.3).

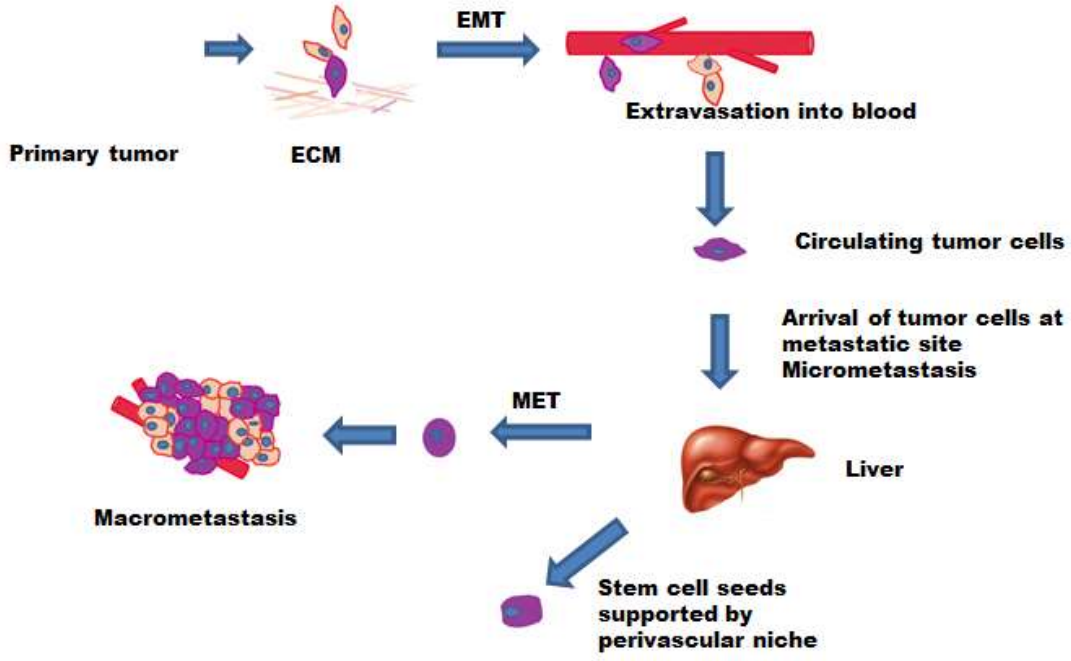


Figure 1.3: Scheme for EMT/MET leading to CRC metastasis. Cells at the primary tumor site invade extra cellular matrix (ECM) and undergo EMT-like program to acquire mesenchymal phenotype. These transformed cells then intravasate into lymph nodes and blood stream and then extravasate into foreign microenvironment (liver in this case). There, they may either stay in an EMT/MET equilibrium thereby generating cancer stem cell-like populations or they reestablish primary tumor phenotype via MET-like program at distant organs. Modified after Samatov et al. and Ramakrishna et al. [71, 72]

1.6 Current therapeutic scenario for CRCs

The traditional treatment regimen for CRC is based on the identification of the stage of the disease and then performing surgery followed by adjuvant therapy. Surgery (Colectomy) remains the first line of treatment for all CRC patients and is most efficient and curative for

early stage CRC's that have not spread outside the colon wall (stage 0-1). Patients with early, localized stage of CRC have a five-year survival rate of around 90% [73]. Unfortunately, like other cancers, CRC manifests itself only when the disease begins to progress and is thus diagnosed at later stages. For stage II patients, when cancers have grown through the inner wall of the colon and have extended to the nearby tissue, surgery is usually followed by adjuvant chemotherapy with 5-Fluorouracil (5-FU) and Leucovorin (alone) or Capecitabine. 5-FU is a pyrimidine analogue which gets incorporated into DNA and RNA inducing cell cycle arrest and apoptosis. Capecitabine is a pro-drug which gets converted into 5-FU inside the tissues whereas Leucovorin enhances the effect of 5-FU by inhibiting thymidylate synthetase. In stage III CRC patients, the cancer spreads to the lymph nodes and adjuvant chemotherapy in combination with surgery or radiation is the usual treatment. So far, there are 9 FDA approved anti-CRC chemotherapeutic drugs. However, 5 of the 10 anti-CRC drugs are basic cytotoxic chemotherapeutics that inhibit DNA synthesis and thus attack cancer cells at a very fundamental level, leaving behind strong side effects on normal cells in those patients (Table 1.1).

Table 1.1: Anti-CRC drugs approved by food and drug administration (FDA, USA)

Generic	Type	Target
Capecitabine	Small molecule	DNA synthesis
Fluorouracil	Small molecule	DNA synthesis
Irinotecan	Small molecule	DNA synthesis
Leucovorin	Small molecule	DNA synthesis
Oxaliplatin	Small molecule	DNA synthesis
Regorafenib	Small molecule	RTK
Aflibercept	Recombinant protein	VEGF
Bevacizumab	mAb	VEGF
Cetuximab	mAb	EGFR
Panitumumab	mAb	EGFR

In more advanced cases, when CRC has spread to distant organs in the form of metastasis, stage IV, the five-year survival dramatically drops to 12% emphasizing the need to design therapies which inhibit metastasis [73]. Combination treatment regimes which include chemotherapy/radiotherapy with targeted molecules (Bevacizumab, Aflibercept, Cetuximab, Panitumumab, Regorafenib) are routinely followed in such cases. Bevacizumab or Aflibercept directed against the vascular endothelial growth factor VEGF prevents the growth of new blood vessels to the tumor. Cetuximab or Panitumumab directed against the EGF

receptor is used to block mitogenic factors that promote cancer growth and Regorafenib targeting angiogenic, stromal and oncogenic receptor tyrosine kinase (RTK), are used to treat metastatic CRCs (Table 1.1). However, CRC remains a lethal disease since global mortality burden from CRC primarily caused due to metastasis is still very high. Majority of the patients do not fully benefit from these treatments. Moreover, the current treatment options do not work for every patient because cancers are highly multifactorial and heterogeneous in nature. Notably, current chemotherapeutics generically target the growth of cancer cells and do not target the various phases of metastasis or cancer stem cells either, as they may reside in protective niches within their metastatic sites. Therefore, identification of new generation chemotherapeutics as well as better prognostic and predictive markers for determining the relevant therapy remains indispensable.

1.7 Prognostic and predictive markers of CRC

Prognostic markers are those that define the clinical outcome of a patient in the absence of treatment thereby providing the natural history of the disease. Such prognostic markers are helpful for identifying patients with cancer who are at high risk of metastatic relapse regardless of the treatment. Validation of prognostic factors is relatively straight forward from the statistical point of view. A control group from a randomized clinical trial is an ideal setting for evaluating the prognostic significance of a biomarker [74]. Predictive markers, on the other hand, are those that define the clinical outcome of the patient in response to a particular treatment. Thus, predictive biomarker helps in identifying subpopulations of patient who are most likely to respond to a given therapy. Such marker forms basis for personalized or tailor made therapy. Predictive biomarker validation requires more extensive data for validation, specifically large randomized clinical trials and meta-analysis [75]. One of the very effective validation designs is an ‘interaction’ design wherein, patients are classified according to biomarker level and then randomized to one of two treatments [76]. Moreover, predictive markers might also be used as target for therapy. CRC unfortunately is lagging behind other cancers in terms of clinically established prognostic marker but the growing knowledge and well-designed trials in the recent years promise to fulfill this existing gap. A brief summary of promising prognostic and predictive markers in CRC for clinical application is described as follows.

1.7.1 KRAS

KRAS mutations, mostly in codon 12, 13 and to a lesser extent in codon 61 are mostly associated with CRC outcome. These mutations result in constitutive activation of RAS and

subsequent activation of MAPK signaling pathway, occurring in about 40% of CRCs [26]. The relevance of KRAS as an independent prognostic marker is highly conflicting. According to the Kirsten Ras in colorectal cancer collaborative group study (RASCAL) with retrospective data analysis from 3439 patients, only glycine to valine substitution at codon 12 (identified in 8.6% of the cases) was associated with poor prognosis. This particular mutation was found to have stronger impact on FFS (failure free survival) and OS (overall survival) in Duke's C patients [77]. More recently, however, KRAS mutation status has emerged as a predictive marker for Anti-EGFR based therapy system. 99% of the patients with mutated KRAS do not respond to EGFR inhibition indicating high negative prediction value of KRAS [78]. However the response rate for KRAS wild type patients to EGFR inhibition is only approximately 20%, suggesting the scope to understand the molecular mechanism corresponding to EGFR inhibition resistance in wild type KRAS patients [79].

1.7.2 BRAF

BRAF is a downstream molecule of KRAS in the RAS/RAF/MEK/ERK pathway. Although more than 40 somatic mutations in the BRAF kinase domain have been described, the key mutation in CRC is the classic GTG → GAG substitution at the position 1799 of exon 15 (V600E) [80]. Mutations in BRAF and KRAS are mutually exclusive in colon cancer and BRAF mutations are most common in CIMP+ and MSI-H genotype [81, 82]. A study from 106 stage II patients and 258 stage III patients, BRAF mutation was associated with worse OS in a multivariate analysis [83]. Another study with 1564 patients with completely resected stage II and stage III colon cancer, demonstrated that BRAF V600E predicts poor prognosis in microsatellite stable CRC's [84] . In the same study, the group reported BRAF V600E mutation to be prognostic for OS, especially for MSI-low and MSS patients but not prognostic for relapse free survival (RFS). In contrast, BRAF V600E mutation in MSI-H tumors was found to have no impact on OS [85]. BRAF V600E mutation thus partially predicts resistance to EGFR inhibitors in patients with wild type KRAS. However, interestingly, a big cohort of patients (41%) with wild type KRAS and BRAF did not respond to anti-EGFR based treatments, emphasizing the need for further elucidation of biomarkers in this pathway [86].

1.7.3 Genomic and epigenomic instability (CIN and MSI)

The alternate pathway for colorectal carcinogenesis is either by more common CIN (or aneuploidy), or through MSI (microsatellite instability). CIN is defined as the presence of multiple structural or numerical changes in chromosomes of tumor cells whereas MSI is defined as tumors with instability in at least two of the five microsatellite markers [87] as explained previously. The prognostic value of CIN and MSI is no longer in question because

of large meta-analyses demonstrating that patients with CIN+ disease have a poorer prognosis and patients with MSI+ CRC have a better prognosis then CIN- and MSI- CRCs respectively [88-90].

In addition to being a prognostic marker, several studies have emphasized the predictive value of MSI status determining the response to 5-flourouracil (5-FU)-based chemotherapy [91, 92]. There have been reports of negative prediction value of MSI+ CRC to adjuvant 5-FU therapy [91]. On the contrary, other retrospective study suggest that 5-FU treatment is effective in stage IV MSI+CRC patients [93]. In addition, there have been reports suggesting that MSI+ CRCs are more sensitive to Irinotecan (inhibits unwinding of DNA by inhibition of topoisomerase 1) based therapy [92, 94].

Additionally, Paclitaxel (a well-established mitotic inhibitor from taxane family used as a chemotherapeutic drug for breast, lung and ovarian cancer) sensitivity is based on chromosomal segregation in diploid cells, therefore CIN could also be a negative predictive marker for response to taxanes [95, 96] . Such studies are currently underway. Nevertheless it is reasonable to look for CIN and MSI status to stratify patients and meanwhile more retrospective investigation needs to be done to validate the predictive value of MSI and CIN.

1.7.4 PIK3CA

Phosphatidylinositide-3-kinases (PI3K) are kinases involved in various biological process including proliferation, differentiation, survival, motility which in turn are involved in cancer [97]. 10% to 20% of colorectal tumors have been reported to have PIK3CA mutations [98]. Activation of these pathways by mutations in KRAS, BRAF, and/or PIK3CA is an established mechanism that drives colorectal carcinogenesis [99]. The majority of activating PIK3CA mutations map to 3 sites: exon 9, codons 542 and 545 in the helical domain, and exon 20, codon 1,047 in the kinase domain, with exon 9 and exon 20 mutation being the hotspot targets. Within exon 9 and 20, the most common transversion are c.1624G>A (E542K) and c.1633G>A (E545K) in exon 9 and c.3140A>G (H1047R) in exon 20 [100].

The prognostic value of PIK3CA mutations in CRC for patient survival remains unclear. Various studies have established the role of PIK3CA as a promising prognostic marker for poor survival [101, 102]. However a study from 1,170 CRC patients demonstrates that patients with concomitant PIK3CA mutation in both exons 9 and 20 are associated with worse survival and individual mutation has no significant association with patient survival [103]. They also showed no significant interaction of PIK3CA mutation with BRAF or KRAS mutation in survival analysis. Meanwhile, there is a recent study

suggesting no significant addition of prognostic information from mutations in PIK3CA [104]. Despite all the controversies, PIK3CA mutation was shown to be a very promising predictive marker for adjuvant aspirin therapy. Regular use of aspirin after diagnosis was associated with superior clinical outcome and better survival in patients with mutated-PIK3CA colorectal cancer, but not among patients with wild-type PIK3CA cancer [105]. Another study from European retrospective consortium reports a PIK3CA mutation frequency of 14.5% in their dataset and exon 20 mutation in this gene was associated with a lack of response to Cetuximab in the KRAS wild type cohort [106]. Increased predictive power of PIK3CA mutation in KRAS wild type dataset has been validated in a recent study as well [107] indicating that stratifying the patients with PIK3CA mutations, together with KRAS mutations, might be of benefit in clinical practice.

1.8 MACC1 - A newly identified prognostic and predictive marker for CRC

Our group identified a promising prognostic marker called Metastasis associated in colon cancer 1 (MACC1) through a genome-wide expression analyses carried out on primary, and metastatic tissues and normal mucosa of subjects with CRC.

MACC1 mRNA expression in primary tumors directly correlated with metastasis formation and metastasis-free survival within a 12 year follow up. The five-year-survival rates dropped to 15% for patients with high MACC1 compared to 80% for patients with low MACC1 in their primary tumors [108]. MACC1 not only can be an effective biomarker for malignant tissues, but may also be helpful in differentiating high grade from low grade adenoma [108]. Further there have been numerous follow up studies confirming the prognostic value of MACC1 in metastasis and CRC recurrence [109-111]. MACC1 expression was also found to be correlated with relapse-free survival in patients with rectal cancer treated with chemo- and radiotherapy followed by surgery [112]. Apart from colon cancer, there have been numerous studies suggesting the importance of MACC1 as a biomarker in gastric, pancreatic, hepatobiliary, lung, ovarian, breast and glioblastoma cancers [113]. Recently our group also demonstrated diagnostic and prognostic power of MACC1 circulating transcripts in patient's plasma for predicting metastasis and overall survival [114]. Also in an integrative marker analysis study for KRAS, BRAF, MSI, SASH1 and MACC1 from stage II colon cancer patients, MACC1 outperformed all others and was the only independent parameter for predicting cancer recurrence [115]. Another study from patients with advanced hepatocellular carcinoma, high expression of MACC1 mRNA and the nuclear protein in the tumor predicted poor outcome of cryotherapy in these patients [116]. Also in liver metastases samples of colon cancer, MACC1 predicts further recurrence of the disease [117]. SNP rs1990172 in the

intronic region of MACC1 is demonstrated to be a predictor for reduced overall survival in CRC patients [118]. Another SNP rs975263 in the coding region of the MACC1 gene was suggested to be associated with a shorter metastasis-free survival, especially for stage I or II patients and those who are younger than 60 years of age [119].

Taken together, MACC1 harbors a great potential to be used in clinics as a prognostic marker for the identification of high-risk patients and metastasis and can be used to predict cancer recurrence and therapy response. The following sections will describe in detail about MACC1 and the ongoing research focused on the tremendous potential of MACC1 in CRC carcinogenesis.

1.8.1 The new gene MACC1: Discovery

Going back to 2009, using differential display RT-PCR with colon mucosa, primary tumors and metastases of subjects with colon cancer, MACC1 was identified with no similarity to known genes [108]. The MACC1 gene is located on human chromosome 7 (7p21.1) on the minus strand. Interestingly, the nearest neighbor of MACC1 on chromosome 7p arm are TWISTNB, TWIST1 and ITGB8 which are known to be involved in tumorigenesis and metastasis of CRC [120, 121]. Among distant neighbors are genes for Met and HGF on the 7^{th} chromosome but 7q31.2 and 7q21.1 respectively. The unspliced primary MACC1 transcript contains seven exons and six introns with a longest spliced mRNA of length 3,188 nucleotides (GeneBank: AK131400.1). MACC1 encodes for a protein of 852 amino acids. The N-terminal region constituting 103 to 150 bp contains clathrin box, two Epsin 15 Homology motif (EH) interacting sites, and an adaptor protein 2α (AP2α) binding site. Followed by this is a ZU5 domain involved in mediating protein-protein interaction. The remarkable C terminus possesses both an SH3 binding motif and a variant SH3 domain showing that this combination might result in the formation of homomeric or heteromeric dimers [122]. Our group also demonstrated that both the SH3 domain and SH3 binding motif are crucial for Met transcription and for the translocation of MACC1 into the nucleus [108]. In addition, MACC1 also possess two C terminal death domains, giving hints for its role in regulating apoptosis. Moreover, a phage display screening suggests MACC1 as binding partner for the proline-rich motif of human FasL (CD178) via its SH3 domain [123].

As already mentioned MACC1 expression was found to be directly correlated with metastases formation and metastasis-free survival but various other cellular and biological aspects of this gene have been explored and discussed in the next sections.

1.8.2 Role of MACC1 in CRC progression and metastasis

MACC1 has been shown to induce cell migration, invasion, proliferation and colony formation in cell culture based assays from different solid cancer derived cells [108, 124-130]. The first cellular aspect of MACC1 associated metastasis was demonstrated by our group. Receptor tyrosine kinase Met was identified as the transcriptional target of MACC1 (Fig. 1.4) thereby shedding light on metastasis induced by MACC1 via HGF-Met-MAPK pathway [108]. In CRC patient-derived mouse xenografts, MACC1 showed better correlation with aggressiveness and unfavorable pathological parameters as compared with Met. *In silico* analysis based on the transcriptional binding sequence on the Met promoter, putative MACC1 target gene sets which correlate with high metastasis and aggressive tumors were predicted [131]. Further *in vivo* studies with different mouse models showed enhanced tumor growth and liver metastasis on transplanting MACC1 overexpressing tumor cells [108]. Transplantation of cells with high MACC1 expression treated with shRNA MACC1 led to reduction in tumor formation and metastasis in xenografted nude mice and SCID mice [132]. Mutation in SH3 domain of MACC1 resulted in loss of all the biological functions *in vitro* and *in vivo* [108]. Furthermore there have been studies suggesting the association of MACC1 with EMT markers in patients with gastric cancer. The hallmark epithelial markers (E-cadherin and α-catenin) were inhibited by MACC1, whereas mesenchymal markers Fibronectin, MMP2, MMP9, Vimentin and CD44) were shown to be up-regulated and the effects could be reversed with shMACC1 cells [128]. The mesenchymal markers help degrade extracellular matrix (ECM) and facilitate cell migration [133]. Another study from huh7 hepatocellular carcinoma cell line also suggested the association of MACC1 in inducing EMT via up regulating MMP2 and MMP9 [127]. In nasopharyngeal carcinoma, there has been a report showing association of MACC1 with phosphorylated-Akt expression and β-Catenin [129]. A very interesting study demonstrated three fold higher MACC1 in Lgr5-GFP high cells over low cells suggesting the association of MACC1 with intestinal stem cells [134]. However, these findings are still preliminary and need to be further validated in different solid cancer entities. To conclude, within the tumor progression model for CRC, MACC1 inflation at the key step from the benign to the malignant phenotype is strongly responsible for inducing metastasis.

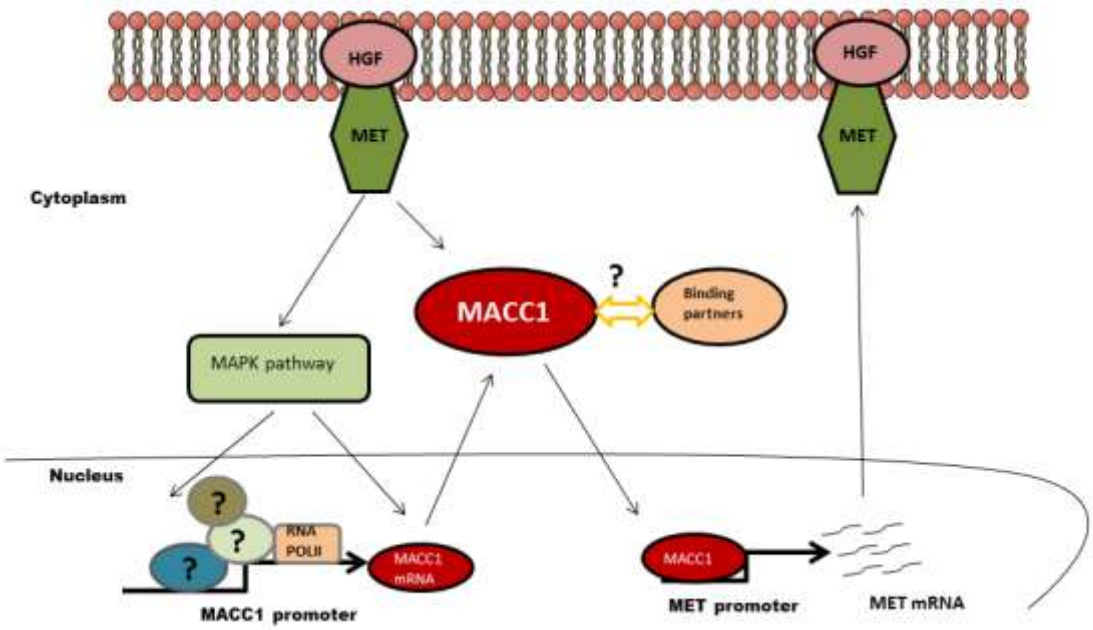


Fig 1.4: Schematic model of MACC1 regulating HGF signaling. HGF translocate MACC1 from the cytoplasm to the nucleus where it binds to the Met promoter. This transcriptional activation of Met, thus forms a positive feedback looping hyper activating HGF-Met pathway and thus metastasis. Furthermore MACC1 is suggested to be a downstream target of MAPK. Adapted from Arlt et al. [135]

1.8.3 miRNAs associated with MACC1

The concept of several noncoding RNA genes, miRNAs, in regulating metastasis is not new. Such miRNAs, termed as metastamir by Hurst et al. contributes to causing lethal metastasis [136]. In a miRNA profiling based study from human CRC's with and without liver metastasis, miRNA signatures were identified classifying patients for metastasis [137]. Further, a study displayed differential expression of miRNAs in CRC's and matched brain metastatic carcinomas [138]. These results suggested that miRNAs might be involved in liver metastasis of human CRC and can be used as prognostic tools as well. The first association of MACC1 with miRNA came from a study describing the role of MACC1 in mir-1 and Met network. They demonstrated that Met is significantly up-regulated by MACC1 only if it is coupled with mir-1 down-regulation [139]. Thus, concomitant mir-1 decrease and MACC1 increase imparts Met associated metastatic behaviour of colon cancer cells. A little later, mir-143 was identified as the first miRNA binding and regulating MACC1 and MACC1-induced cell motility. They reported that mir-143 might function as a metastamir by inhibiting cell migration and invasion via targeting MACC1 [125]. More studies are needed to validate the role of these miRNAs as independent biomarkers or to establish their possible role to enhance the prognostic and predictive potential of MACC1.

1.8.4 Miscellaneous roles of MACC1 apart from cancer

Besides cancer, MACC1 has been proposed to be associated with the development of other non-cancerous pathologies. Data from one group attributed the role of MACC1 to craniofacial development. This group used microarray analysis to catalogue gene transcripts involved in mouse face formation [140]. Among the candidates was the MACC1 gene which was validated using Morpholino (MO) based screening to be involved in a progressive and dose dependent loss of neural crest–derived cartilages [141]. Early in 2005, MACC1 (referred to as 7a5 earlier), was expressed in retinal cells ARPE-19 and Y79 as well as in neural retina and retinal pigments epithelium extracts [142]. There have been clues of MACC1 being responsible for maintaining pluripotency as predicted by neuron derived iPSC's screen using RNA-Seq. [143]. A study on a family from Pakistan with autosomal recessive non-syndromic hearing impairment (ARNSHI) led to the identification of novel locus DFNB90 mapped to chromosome 7 (7p22.1-p15.3). MACC1 was one among the candidate gene which resides in this locus. However on sequencing the candidate genes, no causal variant was identified [144]. Apart from the putative role of MACC1 in development, there might be a possibility of its link to migraine as depicted by a meta-analysis of genome-wide associations with respect to migraine. This analysis reports 32 SNPs with MACC1 being third highest in terms of its p value [145]. Similarly another genome wide screen came up with MACC1 SNPs (rs206184 and rs6974002) to be involved in coronary artery stenosis [146]. In addition MACC1 might be involved in immune/inflammatory processes. MACC1 was found to be amongst 200 gene regions associated with multifactorial Crohn’s disease [147]. Another hint for MACC1 being associated with inflammation was shown in a study which focused on the transcriptional effects of IFN γ or IL-4 treatment on subsequent TLR4 activation in mouse macrophages. MACC1 was one amongst the genes which was cooperatively up-regulated by IL-4 and LPS [148]. All these studies pin down the role of MACC1 in development and immunity in addition to cancer progression. However, more research needs to be done to address these additional features of MACC1. A MACC1 knock out animal if generated in the future might explain these findings.

“Patients rarely die from the effects of a primary tumor; 90 percent of deaths from cancer are the result of metastases, of malignant cellular outposts proliferating far from the neoplastic mass that spawned them. They are barbarians, the colonist cells, co-opting all nutrients in their adopted organ and starving their normal neighbors of air, sugar and salts, and blocking traffic and clogging conduits, and finally, when their greed exceeds their easy grab, tearing open surrounding cells and feasting like cannibals on the meat of their fellows.”

Natalie Angier, New York Times

2. AIM OF THE STUDY

The past few years have established MACC1 as a powerful prognostic and predictive marker for metastasis and metastasis free survival. In addition, these studies have emphasized the role of MACC1 in CRC progression. shRNA against MACC1 was shown to inhibit tumor formation and metastasis in xenografted mice models. Our present study along with the previously known studies, envisioned the importance of MACC1 inhibition. However the protein structure of MACC1 remains unknown and potential MACC1 protein inhibitors could not be generated. Besides, nothing was known about the promoter sequence and transcriptional regulation of this gene and thus no MACC1 transcriptional inhibitor has been reported so far.

Thus, the main objectives of this study are as follows:

- Identification of the MACC1 promoter and elucidation of its transcriptional regulation mechanism. This included identification and validation of the transcription factors binding to the MACC1 promoter. Additionally, to provide proof of principle by showing regulation of MACC1 expression and MACC1 induced motility by these transcription factors and to further address the significance of these transcription factors in tumor specimens with high MACC1 levels and metastasis formation.

- Translation of the knowledge from the promoter studies to identify small molecule transcriptional inhibitor(s) of the MACC1 gene. This included evaluating these inhibitor(s) *in vitro f*or the reduction in MACC1 expression and MACC1 associated motility. Further, the study included validating these inhibitor(s) *in vivo* for their ability to restrict MACC1-induced-tumor progression and metastasis formation in xenografted mice models.

3. MATERIALS AND METHODS

3.1 Cloning

Genomic DNA (gDNA) was isolated from SW620 cells using QIAamp DNA Mini Kit (Qiagen). gDNA was PCR amplified using Pwo master mix (Roche) with a specific set of primers to obtain the desired insert. The PCR product was then purified by ethanol precipitation. DNA (PCR product as well as vector) was digested with FastDigest® Restriction Enzymes in 1x FastDigest® Buffer (both Fermentas) at 37 °C for 1.5 h. Digested DNA was separated by agarose gel electrophoresis at 100 V for 30 min in gels containing 1% w/v agarose (Invitrogen) in TAE-buffer (40 mM Tris, 1 mM Na_2EDTA and 20 mM acetic acid, pH 8). DNA was then purified from the agarose gel using Invisorb® Spin DNA extraction Kit (Invitek) according to the manufacturer's instructions. Purified DNA was ligated in a 1:10 ratio of vector backbone and insert. Ligation was carried out with 0.25 U/µl T4 Ligase in 1x Ligase Buffer (both Fermentas) for 1 hat room temperature. Bacterial transformation was performed in DH5α™ chemically competent cells (Invitrogen) according to the manufacturer's instructions. Transformed bacteria were spread on selective agar plates and allowed to grow overnight at 37°C. DNA plasmid preparation from positive colonies was performed with Invisorb ® Spin Plasmid Mini Two (Invitek). Control digestion of plasmids followed by agarose gel electrophoresis identified positive clones. For preparing plasmids suitable for transfection, endotoxin-free plasmid DNA Maxi Prep was done using the JETSTAR 2.0 Maxi kit (Genomed) according to the manufacturer's instruction. Cloned constructs were sequenced for correct in frame orientation (Stratec Molecular).

3.1.1 MACC1 promoter luciferase reporter constructs

The MACC1 promoter region was PCR amplified using genomic DNA from SW620 cells as a template. Two different promoter fragments -1982 to -18 and -992 to -18 were generated using the primers listed in Table 3.1. PCR was performed using Pwo master mix with an initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 3 min. A final extension step was included at 72°C for 5 min. The PCR product was ethanol precipitated and digested with *SacI* and *BglII* restriction enzymes. The digested product was ran on gel, purified and cloned into *SacI* (5') and *BglII* (3') restriction sites of the pGL4.17 vector from Promega (pGL4.17/MACC1p-992 and pGL4.17/MACC1p-1992). All the restriction enzymes were fast digest enzymes from Fermentas. Control digests of plasmids with *SacI* and *BglII* identified positive clones. Plasmids were purified and sequenced as described in section 3.1.

3.1.2 5'-deletion constructs of MACC1 promoter

A series of different 5'-truncated fragments of this -992 to -18 promoter region was created using different primers mentioned in Table 3.1 and cloned into the pGL4.17 vector using restriction enzymes (*SacI, EcoRV and BglII*), resulting in pGL4.17/MACC1p-811, pGL4.17/MACC1p-582, pGL4.17/MACC1p-426, pGL4.17/MACC1p-206 and pGL4.17/MACC1p-133. All constructs were sequenced as described earlier.

Table 3.1: Primers used for generation of the MACC1 promoter-luciferase clones

Primer	*Sequence*
pGL4.17/MACC1p$_{-1992}$ F	5'-ACCGGAGCTCAGAAGGGATCCATAAATGCTGG-3'
pGL4.17/MACC1p$_{-992}$ F	5'-ACCGGAGCTCTTTTTCTTTGTCACCATTCTGCCA-3'
pGL4.17/MACC1p$_{-811}$ F	5'- ACCGGAGCTCCTCTGTTGCTGATGTTGGAAA -3'
pGL4.17/MACC1p$_{-582}$ F	5'- GGT TGATATCAATCCAGAGCATTTTAGAAGATG-3'
pGL4.17/MACC1p$_{-426}$ F	5'- GGTTGATATCAGGGCAGTGAGGCACCTT -3'
pGL4.17/MACC1p$_{-206}$ F	5'- ACCGGAGCTCTAATTGCTTTTCCACCTGCTTC-3'
pGL4.17/MACC1p$_{-133}$ F	5'- GGTTGATATCGTGAGGAGCCTGAATTGTG-3'
pGL4.17/MACC1p$_{-1992}$ R	5'-GCTTAGATCTCCCTGCTTCCTGAGCCAC-3'
MACC1p$_{-426}$ mut c-Jun F	5'-CTTCAGCTCTGAAT**TG**CCGAAAGAGAATCT-3'
MACC1p$_{-426}$ mut c-Jun R	5'-AGATTCTCTTTCGG**CA**AT TCAGAGCTGAAG-3'
MACC1p$_{-426}$ mut Sp1 F	5'-ACTCTAGCC ATACGC**AA**TCTTCTGGTT TCG-3'
MACC1p$_{-426}$ mut Sp1 R	5'-CGAAACCAGAAGA**TT**GCGTATGGCTAGAGT-3
MACC1p$_{-426}$ mut C/EBP F	5'-GCTGCATGAGGATTTGC**GG**GCATAAATATTTTTTAC-3'
MACC1p$_{-426}$ mut C/EBP R	5'-GTAAAAAATATTTATGC**CC**GCAAATCCTCATGCAGC-3'

Underlined bold base pairs represent the nucleotides which have been mutated to generate mutated MACC1 promoter fragments.

3.1.3 Site directed mutated constructs of the MACC1 promoter

Site directed mutagenesis was performed using the QuikChange site directed mutagenesis kit (Stratagene) according to the manufacturer's instruction. The -426 to -18 reporter construct was used as template and a two base pair mutation in the binding site of each transcription factor AP-1, Sp1, C/EBP was inserted using primers with the mutated sequence

as underlined and highlighted in bold, Table 3.1. Plasmids with the expected mutations were sequenced to confirm the existence of the mutation.

3. 2 Cell culture

3.2.1 CRC cell lines

Cell culture media, PBS and Trypsin/EDTA solution were obtained from Life Technologies. Cell culture plastic ware was obtained from TPP, BD Biosciences or Greiner BioOne. All human CRC cell lines originally from American Type Culture Collection used in our study are listed in Table 3.2. Cells were grown in RPMI-1640 (PAA Laboratories) supplemented with 10% fetal bovine serum (FBS; Invitrogen). All cells were maintained at 37^0C in a humidified 5% carbon dioxide incubator. Cells were trypsinized and split in a 1:4 ratio every 3-4 days. All cells were negative for mycoplasma, verified regularly using MycoAlert® Mycoplasma detection kit (Lonza) Authentication of the cell lines was performed by short tandem repeat (STR) genotyping at the Leibniz-Institut DSMZ (Braunschweig, Germany). STR genotypes were consistent with published genotypes for these cell lines.

Table 3.2 Summary of all colorectal cancer cell lines used in this study

Cell line	Medium	ATCC number
Caco2	DMEM, 10 % FBS	HTB-37
DLD1	DMEM, 10 % FBS	CCL-221
SW48	RPMI 1640, 10 % FBS	CCL-231
SW480	RPMI 1640, 10 % FBS	CCL-227
SW620	RPMI 1640, 10 % FBS	CCL-228
HCT116	DMEM, 10 % FBS	CCL-247

All cell lines are registered in the American type culture collection (ATCC).

3.2.2 Derivative cell lines

HCT116-MACC1p-Luc cells used in the high throughput screening were obtained by stable transfections of MACC1 promoter constructs in HCT116 cells. Briefly $1x10^6$ cells were plated in a 10 cm dish. For each transfection, 5 µg of pGL4.17/MACC1p$_{-992}$ orpGL4.17 empty plasmid and 15 µl Fugene HD (Roche) were incubated for 20 min in 500 µl OptiMEM at room temperature. Subsequently the transfection mixture was added to the cells of 70% confluence in a total volume of 10 ml DMEM medium. After 48 h, selection of positive

transfected cells occurred by treating cells with 1 mg/ml neomycin. Antibiotics were continuously present and were removed only 24 h before the experiments to avoid their interference.HCT116-CMVp-Luc cells applied in the high throughput screening and for *in vivo* imaging experiments were obtained by transfection of HCT116 with pcDNA3.1-puro-Luc. Stable expression of transgene was controlled regularly by Steady Glow^{TM} Luciferase Assay System (Promega) according to the manufacturer's instructions.

SW620-shControl and SW620-shGIPC1 cells with stable knockdown of GIPC1 were obtained by transfection of sh Control and shGIPC1 plasmid (SABiosciences) in SW620 cells and positive clones were selected using neomycin treatment. Stable knock down was monitored via regular detection of GIPC1mRNA levels by qRT-PCR. All the derivative cell lines are mentioned in Table 3.3 and the plasmids used for generating these derivative cell lines are described in Table 3.4

Table 3.3 Summary of derivative cell lines used in this study

Cell line	***Medium***
HCT116-pGL4.17-Luc	DMEM, 10 % FBS
HCT116-MACC1p-Luc	DMEM, 10 % FBS
HCT116-CMVp-Luc	DMEM, 10 % FBS
SW620-shControl	RPMI, 10 % FBS
SW620-shGIPC1	RPMI, 10 % FBS

3.3 Transfections and drug treatment

3.3.1 MACC1 promoter transfections

Transient transfections were carried out in 24 well culture plates using Fugene HD (Roche) as a transfection reagent. 75000 cells were plated per well. For each transfection, 1 µg of pGL4.17 vector consisting of a MACC1 promoter fragment or pGL4.17 empty vector along with 100 ng of pGL4.74 Renilla luciferase plasmid (internal control) and Fugene were incubated for 20 min at room temperature, in 100 µl OptiMEM. This transfection mixture was then added to the cells of 70% confluence in a total volume of 500 µl RMPI 1640 medium. DNA to lipid ratio of 1:3 was used for all the experiments.

Table 3.4 Plasmids used for colorectal cancer cell transfection

Plasmid	*Features*
pGL4.17-MACC1p-Luc	MACC1 promoter driven firefly luciferase cDNA; neomycin resistance
pcDNA3.1-puro-Luc	CMV promoter-driven firefly luciferase cDNA; puromycin resistance
pcDNA3.1-MACC1	CMV promoter-driven MACC1 cDNA; neomycin resistance
pGeneClip-shGIPC1	U1 promoter driven shGIPC1; neomycin resistance

3.3.2 siRNA transfections

Predesigned siRNA's for c-Jun and Sp1 were obtained from Ambion, whereas for C/EBPα and C/EBPβ, siRNA's were obtained from Qiagen. For RNA interference experiments, $2x10^5$ HCT116 cells were seeded in a 6 well plate. After 24 h, cells were transfected with 50 nM siRNA targeting c-Jun, Sp1 or C/EBP using RNAiMAX lipofectamine transfection reagent (Invitrogen). 50 nM siRNA was diluted in 250 µl of OptiMEM and was gently mixed with 250 µl of OptiMEM containing 7 µl of lipofectamine. The transfection mix was incubated for 20 min at room temperature and then was added to the plate containing 2 ml of media.

3.3.3 Drug treatment

The small compound inhibitors Rottlerin and Lovastatin were obtained from Santa Cruz Biotechnology and stored in dark at -20°C. Both drugs were solubilized in dimethylsulfoxide (DMSO) for *in vitro* application. The stock solution of 10 mM was prepared fresh every two weeks and stored in small aliquots at 20°C to avoid freeze thawing. To exclude adverse effects caused by DMSO, control cells were always treated with the equal amount of solvent. *In vivo*, Rottlerin was administered as suspension in 1% DMSO in saline + 0.5% Tween80.

3.4 High throughput screening

HCT116 cells stably expressing MACC1 promoter ahead of luciferase gene (HCT116-MACC1p-Luc) were seeded into white 384 well plates (Corning) using the Tecan automatic pipetting system. A compound library consisting of 30,000 compounds from Chembionet was used and then test compounds were added on to the plate containing cells. The treatment was carried out for 24h at a concentration of 5 µM per compound. Following treatment, the luciferase signal was measured using a Tecan microplate reader. In parallel, a selectivity screen to eliminate general luciferase inhibitors, was carried out using HCT116 cells stably transfected with the CMV promoter ahead of the luciferase gene (HCT116-CMVp-Luc cells). All the measurements were made in triplicates. The compounds which were able to reduce

luciferase value by 50% by specifically acting via MACC1 promoter were shortlisted. Compounds showing best evidences for selectivity screen were further used for concentration response testing using triplicate wells per concentration with a concentration range starting from 0.097 µM to 50 µM. High throughput screening and all the parameters used in the screening are explicitly described in Table 3.5.

3.5 Cell culture based *in vitro* assay

3.5.1 Dual Luciferase reporter gene assay

Cells transfected with MACC1 promoter constructs and Renilla plasmid were evaluated for luciferase activity 24 h post transfection using the dual luciferase reporter assay system from Promega. Cells were lysed with 100 µl passive lysis buffer and kept on shaker for 15 min at room temperature. 20 µl of this lysate was then transferred to a white bottom plate and firefly luciferase activity as well as Renilla luciferase activity was measured with a luminometer (Tecan infinite 200 PRO). The firefly luciferase values were normalized with Renilla values which account for variations in transfection efficiency and cell number.

3.5.2 Boyden chamber assay

Cell migration analysis was performed with Boyden chamber assay. Cells were co-transfected with siRNA for c-Jun or Sp1 along with either pcDNA3.1 as vector control or pcDNA3.1/MACC1 using Lipofectamine 2000 for 48 h. The pcDNA3.1/MACC1 plasmid used in our study has been documented earlier [119]. After an overnight serum starvation of cells, the transfected cells ($2.5x10^5$ cells in 300 µl RPMI-1640 with 2% FBS) were seeded into each transwell chamber (Millipore). The transwells were pre-soaked with medium prior to the seeding of cells. Fresh medium was added to the bottom chamber with 10% FBS and cells were allowed to attach and migrate for 24 h. Afterwards cells that had migrated to the lower chamber were incubated in trypsin-EDTA and counted 9 times in Neubauer chambers. For monitoring migration in response to the drug treatment, serum starved HCT116 cells ($3x10^5$ cells in 300 µl of drug containing RPMI-1640 with 2% FBS) were seeded into pre-soaked transwell chambers with pore size of 8 µm (Corning). 650µl of fresh medium with 10% FBS and drug was added to the bottom chamber. The cells that had migrated to the lower chamber were stained with DAPI and 4-5 random pictures per transwell were taken under fluorescent microscope and then the cells were counted manually from those pictures.

Table 3.5: High throughput screening criteria

Category	*Parameter*	*Description*
Assay	Type of assay	Cell based Luciferase Reporter assay
	Target	MACC1 promoter
	Primary measurement	Luminescence
	Key reagents	Steady glow reagent from Protégé
	Assay protocol	1. Day1: cell seeding 5,000 cells/well in 40 µl RPMI medium 2. Day2: add compounds 5 µM (Tecan Wall E, 0.2 µl, fc0.5% DMSO) 3. Day3: - Aspirate with Tecan/WallE_10µl left - Add 10µl steady-Glo luciferase reagent in MACC1_cell platesP1, mix, and centrifuge - Measuring Luminescence 500 ms integration time after 15 min incubation
Library	Library size	30,000 compounds library
	Library composition	Drug like molecules and LOPAC compounds
	Source	Chembionet
Screen	Format	384 well type
	Concentration tested	5 µM
	Plate controls	HCT116-pGL4.17 empty vector cells, and Untreated and DMSO treated cells
	Reagent/ compound dispensing system compounds	5 µM (Tecan WallE, 0.2 µl, fc0.5% DMSO)
	Detection instrument and software	Tecan infinite PRO
	Tecan luminometer at integration time 500m/s	
	Assay validation/QC	Z score > 0.4
	Correction factors	1.48
	Normalization	Percentage of control (POC)
Post-HTS analysis	Hit criteria	Z score
	Hit rate	
	Additional assay(s)	Counter/specificity screen with HCT116-CMVp-Luc cells
	Confirmation of hit purity and structure	Mass spec

3.5.3 Cytotoxicity assay

Analysis of cell cytotoxicity was performed with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma Aldrich) assay. Briefly, $5x10^3$ cells were seeded into 96-well-plates and allowed to adhere to the bottom of the wells for 24 h. The cells were exposed to different concentrations of a compound or its solvent for 24 h. MTT was added to a final concentration of 0.5 mg/ml and incubated for 3 h at 37°C in a humidified incubator.
MTT was reduced to purple formazan crystals by the mitochondria of living cells and the decrease in metabolized MTT represented decreased cell number. Formazan crystals were dissolved in 150 µl of DMSO and the absorption was measured at 560 nm in the absorbance reader (Tecan infinite 200 PRO). Cell viability was determined by dividing the absorbance ratio of formazan crystals of treated cells by the ratio obtained from untreated cells which was defined as 100% cell viability. Each experiment was done at least two independent times, each performed in triplicates.

3.5.4 Wound healing assay

The wound healing assay was used to analyze directed cell migration. On day 0, $5x10^4$ cells were seeded into cell culture inserts (ibidi) to create a wound. After appropriate attachment time (24 h), culture inserts were gently removed using tweezers. A wound of about 500 µm width was inflicted after removal of cell culture inserts. Subsequently, medium containing drug was added. The progress of wound closure was monitored daily and microphotographs of 10x and 40x magnification were taken with the Leica DM IL light microscope (Leica Microsystems) on day 0 and day 3. Each wound healing assay was performed two independent times each time in duplicates.

3.5.5 Colony formation assay

Analysis of anchorage-independent cell proliferation was achieved by soft agar colony formation assay. A bottom layer containing 0.5 % w/v agarose, RPMI 1640 medium, 10% FBS and inhibitor or solvent was added to a 6 cm Ø dish. Onto the solidified bottom layer a top layer was added containing $8x10^3$ cells, 0.33 % w/v agarose, RPMI 1640 medium, 10% FBS and inhibitor or solvent. Cells were seeded as single cells into the soft agar and incubated in a humidified incubator at 37°C and 5% CO_2 for 10 days. Colony formation was visualized by 10x magnification for an overview and 40x magnification for single colonies in the Leica DM IL light microscope (Leica Microsystems). Colony quantification was achieved by counting cell colonies of more than 4 cells in 12 squares of 1 μm^2.

3.5.6 Proliferation assay

For determination of anchorage-dependent cell proliferation, $4x10^3$ cells were plated into 96-well-plates and were allowed to accommodate for 15 h, before treatment was started. Cells were treated daily for 4 days with inhibitor or solvent. For determination of viable cells, MTT was added to a final concentration of 0.5 mg/ml, incubated for 3 h, and dissolved in DMSO and kept for shaking for 15 minutes. The optical density (OD) was measured at 560 nm as mentioned earlier. Each cell proliferation experiment was performed in triplicates.

3.6 Protein-DNA interaction based assays

3.6.1 Chromatin Immunoprecipitation assay

Chromatin Immunoprecipitation assay (ChIP) assay was performed using EZ ChIP™ kit from Millipore as per manufacturer's instruction. All the reagents were provided in the kit unless stated. Cells ($2x10^6$) were plated in 10 cm dishes. After 24 h, the cells were cross-linked with 1% formaldehyde for 10 min at room temperature**,** lysed and sonicated to release chromatin. Cell lysates were sonicated for 25 pulses at 40% output and centrifuged at 10,000 rpm for 10 min. Supernatant was collected in a new tube and diluted using dilution buffer and protease inhibitor. 1% of this solution was stored at 4°C until the elution step to be served as input control.

The protein-DNA complexes were precipitated on addition of polyclonal antibodies for c-Jun (Millipore), Sp1 (Millipore), C/EBPα (Active motif), and C/EBPβ (Santa Cruz) to the chromatin solution obtained above, overnight at 4°C. Protein G beads were then added and incubated for another 2 h at 4°C.Non-bound protein was washed away twice with the Wash Buffers provided in the kit. The protein-DNA-complex was eluted from the beads with the elution buffer followed by centrifugation at 3,000 rpm for 1 min. Cross linking of protein and DNA was reversed at 68°C overnight and residual protein was digested by proteinase K at 55°C for 2 h. DNA was purified by column purification. The extracted DNA was subjected to PCR (28 cycles at 94°C for 30 s and 60°C for 30 s and 72°C for 1 min) with MACC1 promoter primers (pGL4.17/MACC1p-$_{426}$ F and pGL4.17/MACC1p$_{-1992}$ R). The PCR product was run on an agarose gel to visualize the precipitation of the MACC1 promoter. GAPDH was used as an unrelated gene to validate the specificity of binding observed.

3.6.2 Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed using the LightShiftChemiluminescent EMSA Kit from Thermo Scientific as per manufacturer's

instruction. Briefly $2x10^6$ cells were seeded in a 10 cm culture dish and incubated for 24 h for adhering to the surface. Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagent from Thermo Scientific as per manufacturer's protocol. 5'-labeled biotin oligonucleotides for the putative binding sites for AP-1, Sp1 and C/EBP were synthesized (Biotez, Table 3.6) and were annealed by heating both forward and reverse strands for 10 min at 95°C and letting it cool gradually in the water bath.In a total volume of 20 µl, 5 µl of nuclear extracted protein was incubated for 30 min at room temperature along with 0.05 % w/v poly dI·dC, 0.5 mM Tris, 0.05 mM EDTA, 2.5 % v/v glycerol, 0.2 % v/v NP-40, 5 mM $MgCl_2$ and double-stranded biotinylated oligonucleotides containing the respective transcription factor binding site as present in the MACC1 promoter. Electrophoretic separation of the protein-oligonucleotide-complexes was performed in pre-cast Novex 6 % TBE gels (Invitrogen) and in TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) for 60 min at 100 V. Capillary transfer of the protein-oligonucleotide-complexes to the Hybond^{TM}-N nylon membrane (Amersham Biosciences) occurred in 20x SSC buffer (3 M NaCl, 300 mM $Na_3C_6H_5O_7$, pH 7) overnight. Cross-linkage of transferred DNA to the membrane occurred at 250 mJ/cm^2 for 1 min in the FL-20-M FluoLinkCrosslinker (Bachofer). Visualization of biotin-labeled DNA was performed with LightShiftChemiluminescent EMSA Kit (Pierce) according to manufacturer's instructions. For the super shift assay, the respective antibody (described earlier) for the specific transcription factor was added before addition of the corresponding oligonucleotide and incubated for 30 min on ice, whereas 100 fold molar excess of unlabeled oligonucleotides were used in the competition experiments.

3.7 Gene expression analysis

3.7.1 RNA isolation and Reverse transcription

Cells ($3x10^5$) were seeded in a 6 well plate and total RNA was isolated using Roboklon kit from Genematrix according to manufacturer's instructions. RNA was quantified (Nanodrop, Peqlab) and 50 ng of RNA was reverse transcribed with random hexamers in a reaction mix (10 mM $MgCl_2$, 1X RT-buffer, 250 µM pooled dNTPs, 1 U/µLRNAse inhibitor, 2.5 U/µl Moloney Murine Leukemia Virus reverse transcriptase; all from Applied Biosystems). Standard dilutions from the reverse transcription of 250 ng of total RNA isolated from HCT116 cells was used for the standard curve. Reaction occurred at 42 °C for 15 min, 99 °C for 5 min and subsequent cooling at 5 °C for 5 min. Reverse transcripts were either stored at -20 °C or directly used for quantitative real-time PCR.

Table 3.6: Oligonucleotide sequence used for EMSA

Primer	Sequence
AP-1 oligo F	**Biotin**-5´-TCTTCTGGTTTCGGGCAGGGCAGTGAGGCACCTTCAGCTCTGAATCACCGAAAGAGAATC-3'
AP-I oligo R	**Biotin**-5´-GAT TCTCTTTCGGTGATTCAGAGCTGAAGGTGCCTCACTGCCCTGCCCGAAACCAGAAGA-3'
Sp1 oligo F	**Biotin**-5´-CTTCTTAGGGTGAAACTCTAGCCATACGCCCTCTTCTGGTTTCGGGTGAGGAGCCTGAAT-3'
Sp1 oligo R	**Biotin**-5´-ATTCAGGCTCCTCACCCGAAACCAGAAGAGGGCGTATGGCTAGAGTTTCACCCTAAGAAG-3'
C/EBP oligo F	**Biotin**-5´-TGGTGGGGCAAGTTCCAGCTGCATGAGGATTTGCTTGCATAAATATTTTTTACTTATTGC-3'
C/EBP oligo R	**Biotin**-5´- GCAATAAGTAAAAAATATTTATGCAAGCAAATCCTCATGCAGCTGGAACTTGCCCCACCA-3'
AP-1 mut F	**Biotin**-5´-TCTTCTGGTTTCGGGCAGGGCAGTGAGGCACCTTCAGCTCTGAATTGCCgAAAgAgAATC-3'
AP-1 mut R	**Biotin**-5´-GATTCT CTTTCGGCAATTCAGAGCTGAAGGTGCCTCACTGCCCTGCCCGAAACCAGAAGA -3'
Sp1 mut F	**Biotin**-5´-CTTCTTAGGGTGAAACTCTAGCCATACGCAATCTTCTGGTTTCGGGTGAGGAGCCTGAAT -3'
Sp1 mut R	**Biotin**-5´-ATTCAGGCTCCTCACCCGAAACCAGAAGATTGCGTATGGCTAGAGTTTCACCCTAAGAAG-3'

Underlined base pairs represent the nucleotides which have been mutated to generate mutated MACC1 promoter fragments.

3.7.2 Quantitative real-time PCR

The cDNA was amplified using SYBR Green dye chemistry using the light cycler 480 (Roche Diagnostics). SYBR green was obtained from Promega. Primers used for PCR were obtained from Biotez and are listed in Table 3.7. Each PCR reaction was performed in a total volume of 10 µl in 96-well-plates in the LightCycler 480. The PCR protocol for SYBR green based qRT-PCR comprised a pre-incubation step at 95 °C for 2 min followed by 45 cycles of (a) denaturation at 95 °C for 7 sec, (b) annealing at 60 °C for 10 sec and (c) elongation at 72 °C for 20 sec. Each qRT-PCR reaction was performed in duplicate and in parallel cDNA quantification of the housekeeping gene GAPDH was done for normalization. To control for primer dimers or unwanted PCR side products the melting curve was measured with a continuous temperature increase from 65°C to 95°C with a rate of 0.1 °C/sec. Data analysis was performed with LightCycler 480 Software release 1.5.0 SP3 (Roche Diagnostics). Mean values were calculated from duplicate qRT-PCR reactions. Each mean value of the expressed gene was normalized to the respective mean amount of the GAPDH cDNA.

3.7.3 Protein extraction and quantification

For total protein extraction, cells ($2x10^5$) were plated in 6 well plates, and 48 h after siRNA transfection the cells were lysed with RIPA buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, supplemented with complete protease inhibitor tablets; Roche Diagnostics) for 30 min on ice. Cell debris was pelleted at 14,000 rpm for 10 min at 4°C.The supernatant was transferred to a new tube, stored at -80°C or directly subjected to Western blot analysis. Protein concentration was quantified before samples were loaded for Western blot analysis. Quantification was performed with Bicinchoninic Acid (BCA) Protein Assay Reagent (Pierce) according to manufacturer's instructions using 2 mg/ml BSA solution for the standard curve [149]. BCA reaction was incubated at 37°C for 15 min and absorption was measured at 560 nm in the absorbance reader (Tecan infinite 200 PRO).

Table 3.7: Primers used for quantitative real-time PCR

Primer	*Sequence*
MACC1 F	5'-TTCTTTTGATTCCTCCGGTGA-3'
MACC1 R	5'-ACTCTGATGGGCATGTGCTG-3'
GAPDH F	5'-GAAGATGGTGATGGGATTTC-3'
GAPDH R	5'-GAAGGTGAAGGTCGGAGT-3'
c-Jun F	5'-CAGGTGGCACAGCTTAAACA -3'
c-Jun R	5'-GTTTGCAACTGCTGCGTT AG -3'
Sp1 F	5'-GCTCTGAACATCCAGCAAAA -3'
Sp1 R	5'-CAGAGTTTGGAACAGCCTGA -3'
C/EBPα F	5'-CGGTGGACAAGAACAGCAAC3'
C/EBPα R	5'-CGGAATCTCCTAGTCCTGGC-3'
C/EBPβ F	5'-CACAGCGACGAGTACAAGATCC-3'
C/EBPβ R	5'-CTTGAACAAGTTCCGCAGGGTG-3'
β-Catenin-F	5'-GTGCTATCTGTCTGCTCTAGTA-3'
β-Catenin-R	5'-CTTCCTGTTTAGTTGCAGCATC-3'
G6PDH-F	5'-ATCGACCACTACCTGGGCAA-3'
G6PDH-R	5'-TTCTGCATCACGTCCCGGA-3'

Primers were applied to a final concentration of 250 nM.

3.7.4 Western blot analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting was used to analyze protein expression levels. Protein extracts were diluted with PBS to obtain 40 µg of total protein in 1x NuPAGE® loading buffer and 10 % DTT. Protein samples were loaded onto pre-cast NuPAGE®Novex 10 % Bis-Tris Gels (Invitrogen).Protein electrophoresis occurred in running buffer 1x NuPAGE® MOPS at 200 V for 45 min within the XCellSureLock™ Mini Cell System. The pre-stained Spectra™ Multicolor Broad Range Protein Ladder (Fermentas) was used to determine band size.

Semi-dry electrotransfer blotting of proteins onto the Hybond C Extra nitrocellulose membrane (Amersham Biosciences) occurred in blotting buffer (25 mM Tris-HCl, 200 mMglycin, 0,1 % SDS, 20 % methanol, pH7.5) at 25 V for 25 min in the Trans-Blot® Turbo™ Blotting System (Bio-Rad). Quality of the protein transfer was analyzed by protein staining with Ponceau S solution (Sigma). The membrane was washed with TBS-T (50 mM Tris-HCl, 150 mMNaCl, 0.05 % Tween 20, pH 7.5) and blocked for 1 h at room temperature with blocking buffer (5 % milk powder in TBST). Membranes were then incubated overnight at 4°C with primary antibody followed by incubation for 1 h at room temperature with HRP-conjugated secondary antibody, both antibodies diluted in TBST with 5% BSA. Antibody-protein complexes were visualized with WesternBright ECL HRP substrate (Advansta) and subsequent exposure to CL-Xposure Films (Pierce). Immunoblotting for β-actin served as protein loading control.

Table 3.8: Antibodies used for western blot analysis, their dilutions and their origins

Target	Dilution	Antibody
Primary antibodies		
anti-β-Actin	1:10000	mouse monoclonal IgG, Pierce
anti-MACC1	1:1000	rabbit polyclonal IgG, Sigma
Anti-c-Jun	1:1000	rabbit polyclonal IgG, Cell signaling
Anti-Sp1	1:1000	rabbit polyclonal IgG, Cell signaling
Secondary antibodies		
anti-rabbit-HRP	1:10,000	HRP conjugated antibody, Promega
anti-mouse-IgG-HRP	1:10,000	HRP conjugated antibody, Pierce

3.8 Tissue specimens

This study was conducted on tissue specimens from 60 patients with CRC with informed written consent (approved by Charite Ethics Committee, Charite University medicine, Berlin) as described in our previous study [108]. The 60 subjects with stage 1-3 cancer (no distant metastases at the time of surgery) were previously untreated, did not have a history of familial CRC, did not suffer from a second tumor of the same or a different entity and underwent surgical R0 resection (complete resection with no microscopic residual tumor). Thereof, 23 patients developed distant metastases in the following twelve years. The follow-up data of all patients was documented up to 14 years (with a median follow-up of 6.3 years) after diagnosis. Tumors were shock frozen in liquid nitrogen immediately after surgery. Cryosections were performed and every fifth section was stained with hematoxylin. Tumor cell areas were evaluated and marked by a pathologist. Tumor cells were removed from the unstained slides and RNA was extracted by using trizol reagent (Invitrogen) including a DNase step. RNA quality was proven (2100 Bioanalyzer, Agilent), and concentration was measured (RiboGreen RNA Quantitation Kit, Invitrogen).

3.9 *In vivo* tumor formation and bioluminescence imaging

All animal experiments were performed in accordance with the UKCCCR guidelines and in cooperation with Dr. Iduna Fichtner (MDC Berlin, Germany). Further, all animal experiments were approved by the State Office of Health and Social Affairs, Berlin, Germany under the G0289/13permit.

3.9.1 *In vivo* dose-finding for Rottlerin

For dose finding experiments, 6-week-old female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice were randomly assigned to 4 groups with 2 animals per group. Rottlerin treatment was performed daily by intraperitoneal injection (i.p.) of 1, 5 and 10 mg/kg Rottlerin along with the respective amount of solvent as placebo. Body weight was analyzed daily to observe toxic effects of the drug. Mice were sacrificed at day 8.

3.9.2 Intrasplenal tumor transplantation

For intrasplenal transplantation 6-week-old female NOD/SCID mice (EPO GmbH) were used. Mice were anesthetized with 35 mg/kg Hypnomidate® (Jassen-Cilag) and the skin and peritoneum were laterally incised to exteriorize the spleen.HCT116-CMVp-Luccells were resuspended in PBS and kept on ice during the surgery. 3×10^6 of these cells were

intrasplenically injected with a 27-gauge needle. The spleen was carefully placed back, the peritoneum was closed with Surgicryl ® absorbable suture and the skin was clamped twice.

3.9.3 Analysis of metastasis formation *in vivo*

Intrasplenically transplanted NOD/SCID mice were randomly assigned to 2 groups of 4 animals. Treatment of animals started 24 h after cell transplantation. Mice were daily treated intraperitoneally with one dose of 5 mg/kg. Control animals received the corresponding amount of solvent. Mice were sacrificed at day 27 for ethical reasons. Spleen (as the transplantation site) and liver (as a metastasis target organ) were removed. The level of metastasis was evaluated by scoring considering both the number and the size of metastatic nodules (1 mm = score 1; 2 mm = score 8; 3 mm = score 27; 4 mm = score 64; 5 mm = score 125).

3.9.4 *In vivo* bioluminescence imaging

Intrasplenically transplanted SCID mice were randomly assigned to 2 groups of 4 animals each. Mice were treated intraperitoneally with daily doses of 5 mg/kg Rottlerin or solvent. For non-invasive bioluminescence imaging, mice were given Isoflurane gas anesthesia (TH. Geyer) every 3 days and received intraperitoneally 100 mg/kg D-luciferin (Biosynth) dissolved in PBS. Imaging was performed with the VisiLuxx Imager (Visitron Systems) with exposure times of upto 10 min. Metamorph software was used for color coding of the signal intensity (presenting a 256 gray scale) and for generating overlay pictures.

3.9.5 Human MACC1 expression in murine xenograft tissue

Spleen tumor tissue was shock frozen with liquid nitrogen after resection and stored at -80°C. For isolation of mRNA, the tumor tissue was grinded under liquid nitrogen to a fine powder using previously cooled mortar and pestle. RL buffer was added to tissue slices and samples were sonicated for 10 pulses at 40 % output to assure complete cell lysis. RNA isolation and reverse transcription was performed as described in sections 3.7.1 and 3.7.2, respectively. Primers for quantification of MACC1 cDNA were designed to be specific to the human sequence of MACC1 excluding murine MACC1 cDNA to interfere with the measurements.

3.10 Statistical analysis

All calculations and statistics were performed with GraphPad Prism version 5.01. Comparison of two groups was done by Student’s t-test. Comparison of a control versus

several treated groups was performed by one-way analysis of variance (ANOVA) and Bonferroni post hoc multiple comparison. For correlation analysis, non-parametric Spearmen correlation test was used. All significance tests were two-sided, and P values less than 0.05 were defined as statistically significant.

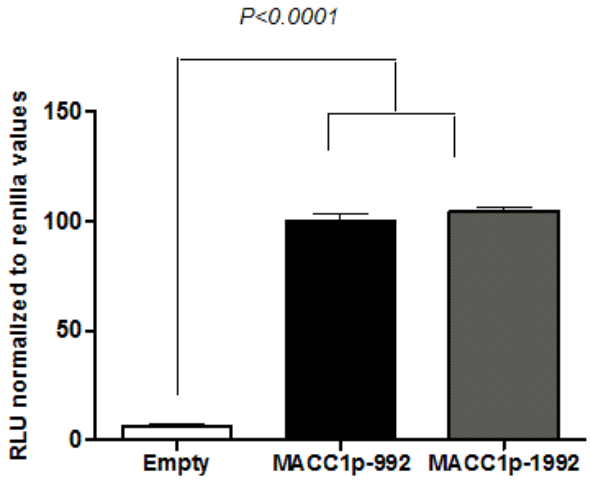
4. RESULTS

4.1 Identification of the MACC1 gene promoter

4.1.1 MACC1 promoter lies within 992 bp upstream of the transcription start site

The transcription start site (TSS) of the MACC1 gene has been already identified by our group using 5' RACE experiment as reported in our previous study [108]. To identify the MACC1 promoter, we cloned sequences upstream of the TSS (+1) of the longest known transcript (-18 to -992 bp and from -18 to -1992 to bp) into the promoter-less luciferase reporter vector pGL4.17. These constructs were then transfected into the HCT116, CRC cell line endogenously expressing MACC1. The promoter activity was measured after 24 h using the luciferase reporter gene assay. As shown in Fig. 4.1A, transfection of these two constructs, MACC1p-1992 and MACC1p-992 into HCT116 cells resulted in significantly higher promoter activity as compared with empty vector control (both P<0.0001) and no significant difference in transcription activity was seen between these two constructs. This finding suggested that the MACC1 promoter region encompassing nucleotides -992 to -18 bp contains all the information to drive transcription of the MACC1 gene in HCT116 cells. This MACC1 gene promoter sequence (Fig. 4.1B) has been submitted to GenBank (GenBank accession code JN544571, 3rd August 2011).

A



B

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-992 TTTTTCTTTGTCACCATTCTGCCATCTACTTACATTAGATGAATCCTTCTATTATGTTTC

-932 TGAACCCAGACCCAGCCAGGACTTGTCTCTATTCATTTTCTGGGCTGTGTCTAACAGGAG

-872 ATAATAGGCTAGAGAGAGATGCTGTATGAACAAATAGAGAAACACATTTGTTTTAAACAT

-812 TCTCTGTTGCTGATGTTGGAAAAAAATGTGAAACAATTATTGCACATTTCATTTCACTAA

-752 GTTTTACCTTTTTTCCCCTTTCCCCTAATTTTCTCTTTCTTGAATTTTGAACAAAATACA

-692 CAGAAGGAAAACACAAAACACAGAAATGGAAAGTAAAATGGAAGAAAATATCAAGAAAAC

-632 TTTATTCTTGCTTATATTTTAAAAGGCACATTTTAAAGTGTTATCTTAAAAATCCAGAGC

-572 ATTTTAGAAGATGAAATGCCAAAAGGTCTCCATTATGTCTATATGTCTATGTCTTTGAGT

-512 GACAATCACAGTGCTGATGTAGAGGGAAAGGGGGAACTAGTTAGACACTGTCACTCACCT

-452 GGGAAGGCTTTATTCACCTGTTCCACAGGGCAGTGAGGCACCTTCAGCTCTGAATCACCG

-392 AAAGAGAATCTGGTGGGGCAAGTTCCAGCTGCATGAGGATTTGCTTGCATAAATATTTTT

-332 TACTTATTGCTAACACTGAGGGTGCCTTCTTACTCCCTGGCAAACATTAAACCACTTTTA

-272 TTTCCTTTCATGGAAATAAGATTATATTTACAGATGGTTCTTAGATATACTCACCTGATT

-212 TTTTTTTAATTGCTTTTCCACCTGCTTCCCCTTTCTTCTTAGGGTGAAACTCTAGCCATA

-152 CGCCCTCTTCTGGTTTCGGGTGAGGAGCCTGAATTGTGGGTATCGTAGTTCTCTTGCCTT

 -92 TTGGGGTTTCTAGTTGGGCAGCTTTGGAGCCACAGTGGTAGAACTTCAAGTCCCAGGTGG

 -32 CTCAGGAAGCAGGG -18
```

Figure 4.1 Identification of the MACC1 promoter. A) MACC1p-1992 and MACC1p-992 luciferase promoter constructs were co-transfected along with pGL4.74 Renilla plasmid in HCT116 cells. After 24 h of transfection, luciferase activity was measured and was normalized to Renilla luciferase activity to correct for the variation in transfection efficiencies. Luciferase activity of the MACC1p-992 construct was set to 100% and activity of the other construct and empty vector alone was calculated and plotted as a percentage of this value. Results are shown as means ± SEM of three independent experiments performed in duplicates. B) Genomic sequence of the region containing the MACC1 promoter. The numbers indicate the base location relative to the start site of the first exon.

4.1.2 MACC1 minimal essential promoter domain lies within 426 bp upstream of the TSS

To identify the minimal essential core promoter region within -992 kb, we cloned six different 5'-deletion constructs of the MACC1p-992 promoter fragment. These constructs were then transfected into HCT116 cells and luciferase activity was measured. Our data (Fig. 4.2 A) demonstrated that deletion of distal promoter fragment from -992 until -426 bp did not decrease the promoter activity significantly. However, further deletion of -426 to -18 bp resulting in -206 to -18 bp and -133 to -18 bp fragments lead to statistically significant decrease in the MACC1 promoter activity by 45% and 81% respectively. These results reveal that the region within -426 to -133 bp incorporate the crucial information to drive MACC1 transcription and regulation and thus represents the core region of the MACC1 promoter. Data obtained in HCT116 cells was validated in an additional CRC cancer cell line, SW620. We transfected pGL4.17/MACC1p-992 andpGL4.17/MACC1p-426 constructs in SW620 and HCT116 cell lines (Fig. 4.2B). We observed no statistically significant differences between the two constructs in both of the cell lines further suggesting that MACC1p-426 construct possesses all essential information for the MACC1 transcription.

Additionally, through ChIP assay, we showed the binding of RNA polymerase II to the MACC1 promoter (-426 to -18) suggesting the presence of the basal transcriptional machinery within the vicinity of this region (Fig. 4.2 C). We also compared human and mouse DNA sequences (http://pipeline.lbl.gov) corresponding to the MACC1 gene and their 5' flanking region. We observed two regions with high degree of conservation (71.4% and 72.1%) immediately upstream of the TSS comprising the core region of the MACC1 promoter, further establishing the importance of that region (Fig. 4.2 D). To summarize, we demonstrated that the MACC1 promoter lies within 992 bp upstream of the TSS. 426 bp upstream of the TSS constitutes the core region of the MACC1 promoter and is sufficient to drive the transcription of this gene. However it was essential to identify this long range promoter (MACC1p-992) as it might encompass trans-acting sequences, enhancer or silencer binding sequences or spatiotemporal information required for tissue specific or developmental stage specific expression of the MACC1 gene.

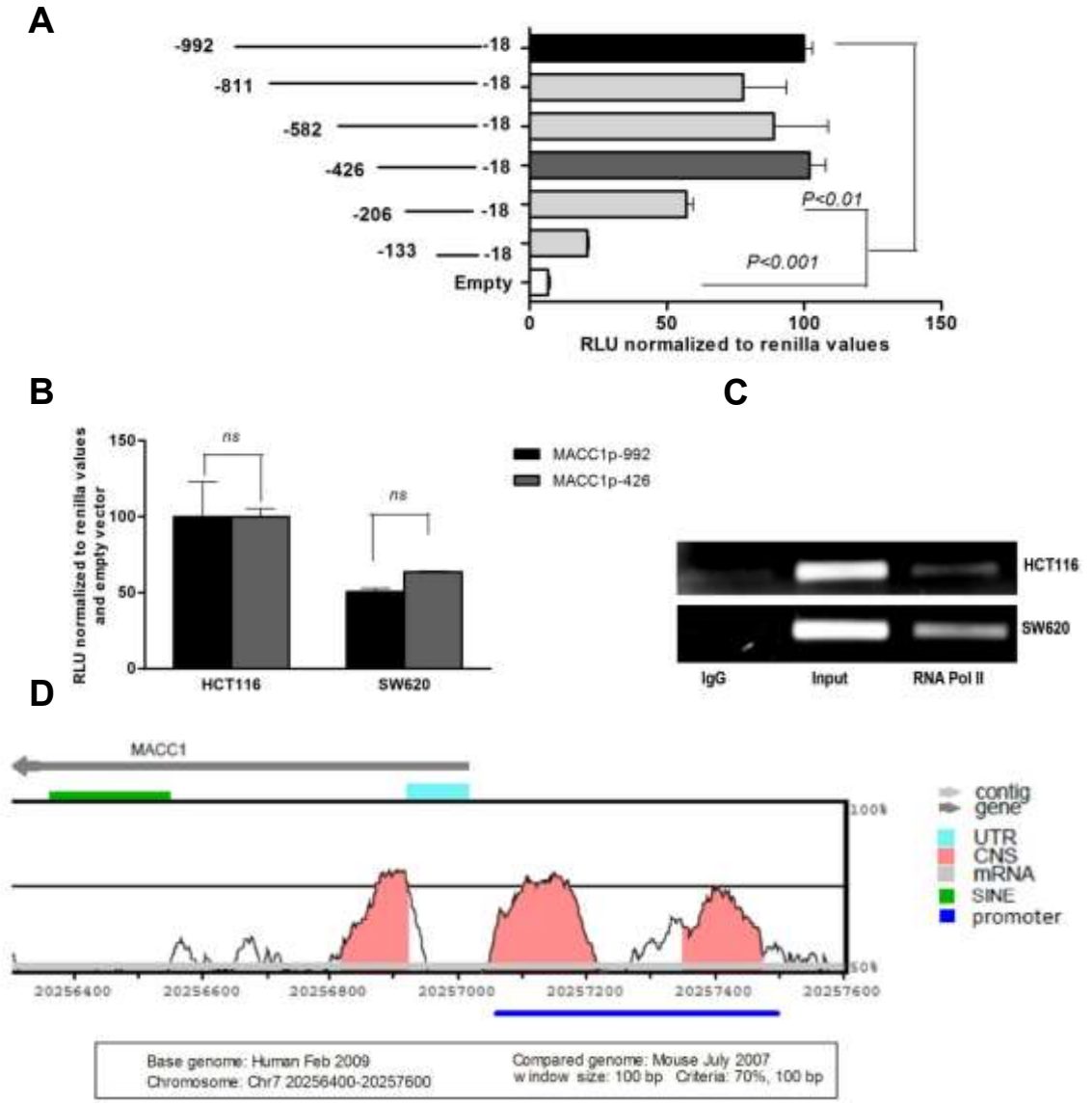
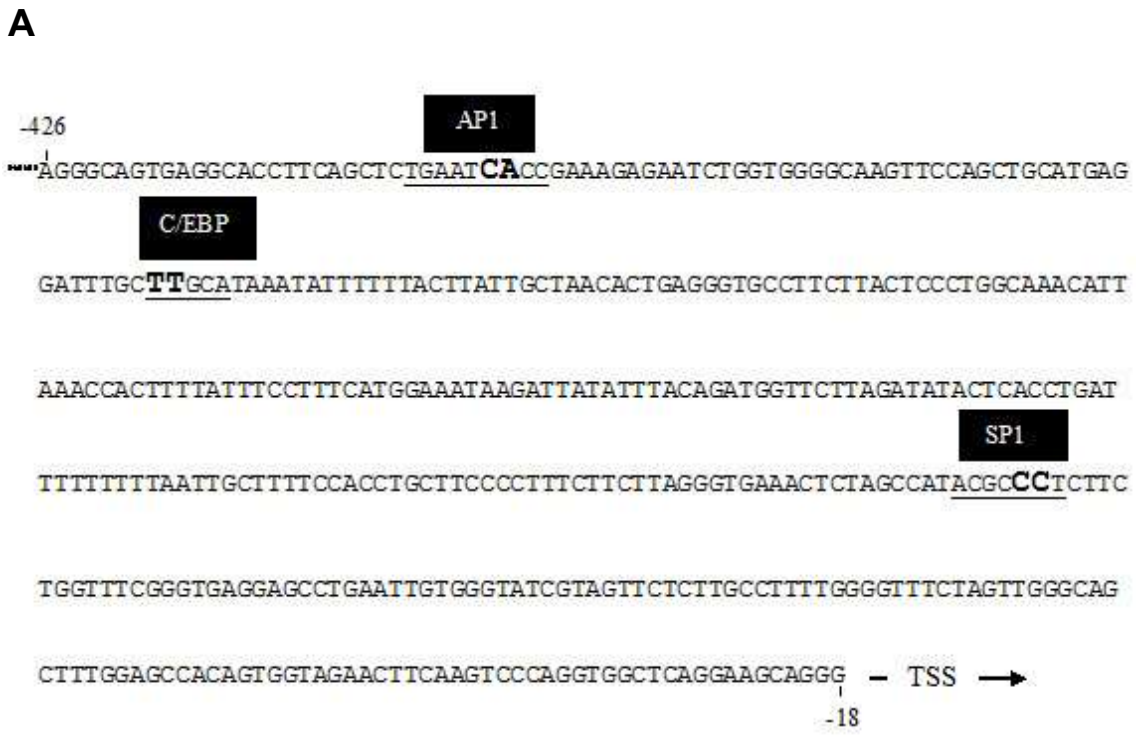
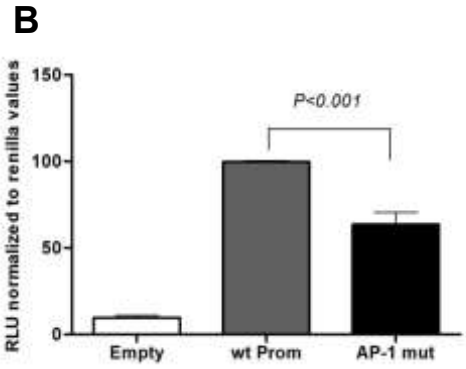


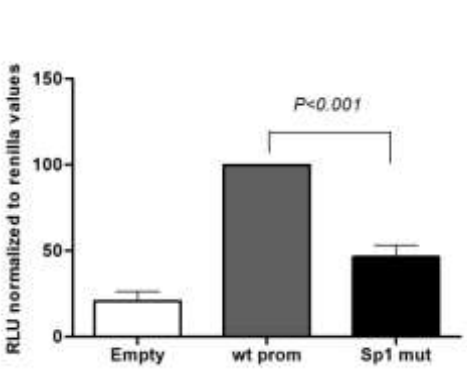
Figure 4.2 Identification of the MACC1 core promoter region. A) Fragments of the MACC1 promoter with deletions at the 5´ end and possessing a common 3´ end were obtained by PCR. The promoter fragments were then inserted into pGL4.17 and were transfected into HCT116 cells. Luciferase activity was measured as described earlier. B) MACC1p-992 and MACC1p-426 constructs were transfected into SW620 and HCT116 cells and luciferase activity was measured. Luciferase activity of the MACC1p-992 construct from HCT116 cells was normalized to empty vector and set to 100% and activity of the other constructs were normalized to empty vector and plotted as a percentage of MACC1p-992 activity. C) HCT116 and SW620 cell chromatin was immunoprecipitated with a RNA Pol II antibody. Non-immune IgG and input DNA without any immunoprecipitation with antibody served as negative and positive controls respectively. After removal of crosslinks, the immunoprecipitated DNA was PCR amplified using a primer set encompassing the basal MACC1 promoter region -426to -18 bp. The amplified DNA was analyzed by agarose gel electrophoresis. Results are shown as means ± SEM of three independent experiments performed in duplicates. D) Human and mouse DNA sequence corresponding to the MACC1 gene and its 5' UTR region were compared. The blue line indicates the MACC1 promoter and the peaks in pink show a region of high degree of conservation between the two species.

4.1.3 The MACC1 promoter harbors functional binding sites for AP-1, Sp1 and C/EBP

To identify transcription factors involved in MACC1 regulation, we performed *in silico* analyses of the MACC1 core promoter sequence to find putative binding sites for specific factors by comparing the results of three different programs: Matinspector (http://www.genomatix.de), Promo Alggen (http://alggen.lsi.upc.es/), and Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess). Examination of the core promoter sequence of MACC1 using *in silico* methods revealed binding sites for several putative transcription factors. We focused on AP-1, Sp1 and C/EBP transcription factor binding sites because of their well-established role in regulating genes involved in carcinogenesis (Fig. 4.3 A). Starting from the TSS, we found a GC box for binding of Sp1. Sp1 has been shown to interact with TATA-binding protein associated factors TAFs and other cofactors, which comprise basal transcription factors [150, 151]. Apart from that, many studies have reported the link between elevated levels of Sp1 in tumors and up-regulation of genes involved in metastasis and survival [152-156]. Furthermore the MACC1 promoter sequence was shown to have a CCAAT box for binding of C/EBPs that can recruit so-called co-activators, such as CBP, that can open up the chromatin structure, or recruit basal transcription factors [157]. Upstream of the CCAAT box, the promoter possesses a binding site for AP1, a well-known transcription factor which is involved in the regulation of genes contributing to differentiation, proliferation and apoptosis [158]. We determined the functional relevance of these DNA binding sites in the MACC1 promoter by mutational analysis. By mutating two bases (shown in bold, Fig. 4.3 A) of each transcription factor binding site, we detected a significant decrease in the MACC1 promoter activity. Mutation of AP-1, Sp1 or C/EBP sites reduced the activity of the MACC1 promoter to 63%, 46% and 55% respectively (all $P<0.001$) (Fig. 4.3 B, C and D), indicating the importance of these sites for the promoter function. Taken together, the MACC1 promoter is a eukaryotic promoter bearing typical promoter elements like a GC box and a CCAAT box along with an AP-1 binding site contributing together for the transcriptional activation of the MACC1 gene.







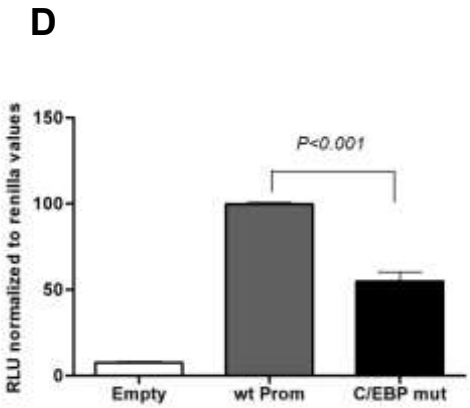


Figure 4.3 Identification of functional binding sites for AP-1, Sp1 and C/EBP. A) Schematic representation of the MACC1 core promoter (-18 to -426 bp upstream of TSS) is shown. The MACC1 promoter harbors binding sites for various transcription factors shown in boxes as predicted by *in silico* programs. B),C),D) Transcription factor binding sites were mutated at two base pairs as shown in bold in (A) and the mutated promoter plasmids were transiently transfected along with the Renilla plasmids into HCT116 cells. After 24 h of transfection, luciferase activity was determined and normalized to Renilla values. Luciferase activity of wt construct was set to 100% and the activity of the other constructs were calculated and plotted as a percentage of this value. Empty represents pGL4.17 plasmid without promoter; wt Prom represents wild type MACC1 promoter and mut represents mutated MACC1 promoter.

4.1.4 AP-1, Sp1 and C/EBP physically bind to the MACC1 promoter

We next performed EMSA experiments to establish the binding of these transcription factors to the MACC1 promoter. Nuclear extracts from HCT116 cells were prepared and incubated with biotin labeled MACC1 promoter oligonucleotides. Binding for AP-1 (Fig. 4.4 A), Sp1 (Fig. 4.4 B) and C/EBP (Fig. 4.4 C) was analyzed by gel shift assay. The biotin labeled oligonucleotides harboring AP-1, Sp1 or C/EBP binding sites were able to form complexes with their respective proteins from the nuclear extract. On further addition of unlabeled oligonucleotide, we observed disappearance of the shifts as evidenced by the ability of excess unlabeled probe to compete with complex formation. The specificity of band was further confirmed by addition of antibodies for c-Jun (is a constitutively active component of the AP-1), Sp1, C/EBPα and C/EBPβ eventually leading to disappearance of the shift. In addition, disappearance of complex formation using mutated oligonucleotides bearing two base pair mutation in the binding region further authenticated the specificity of the binding. These results have also been validated in SW620, another CRC cancer cell line.

We further analyzed the physical interaction of the transcription factors AP-1, Sp1 and C/EBPs with the MACC1 promoter by ChIP assay. A clear band on amplification of immune precipitated chromatin through specific antibody with primers for the MACC1 promoter demonstrated that c-Jun, Sp1, C/EBPα and C/EBPβ bind to the MACC1 promoter (Fig. 4.4 D). Taken together, the results from both ChIP and EMSA analyses in two different cell line models confirmed the physical binding of the transcription factors AP-1, Sp1 as well as C/EBPα and C/EBPβ to the MACC1 promoter.

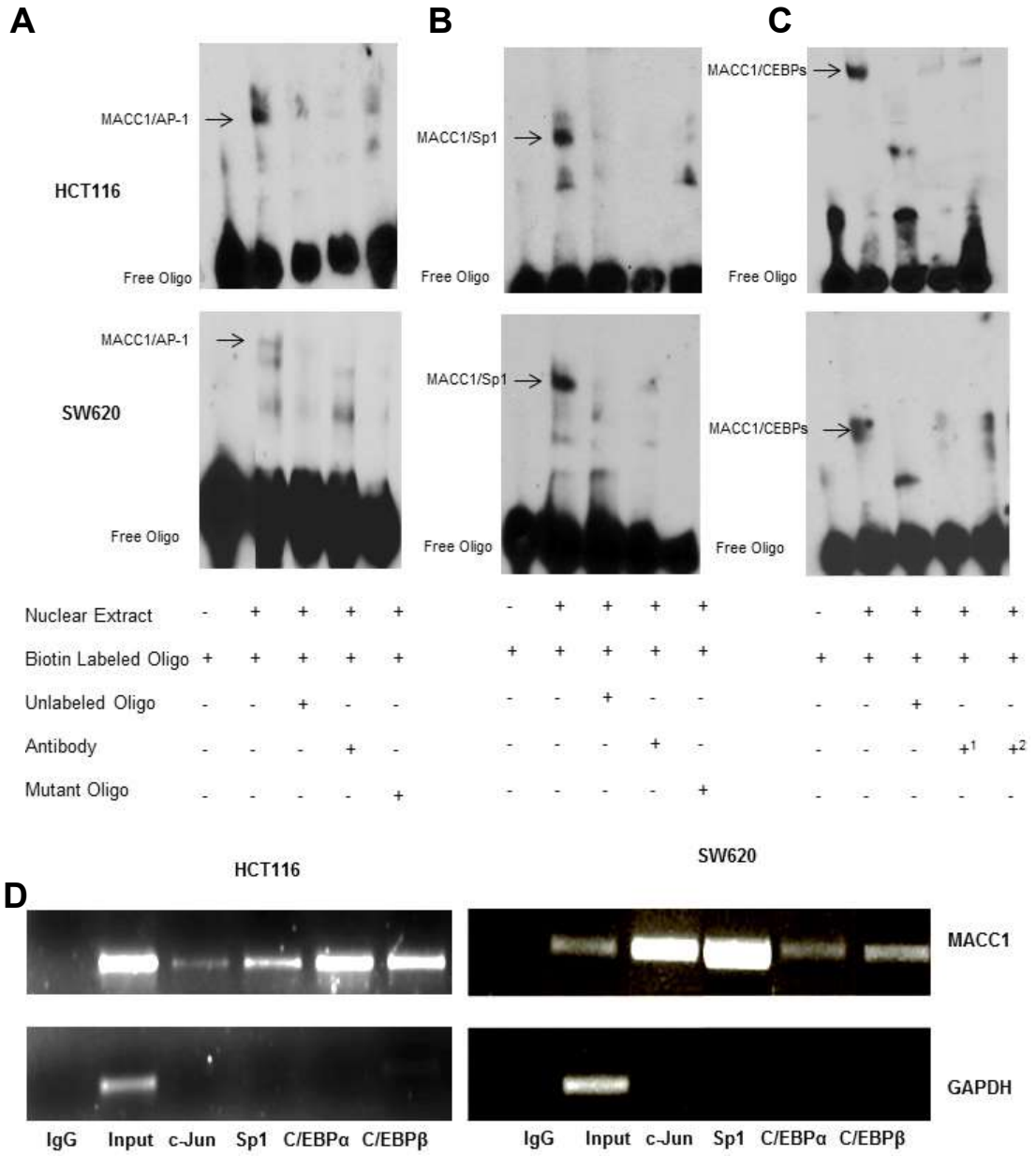
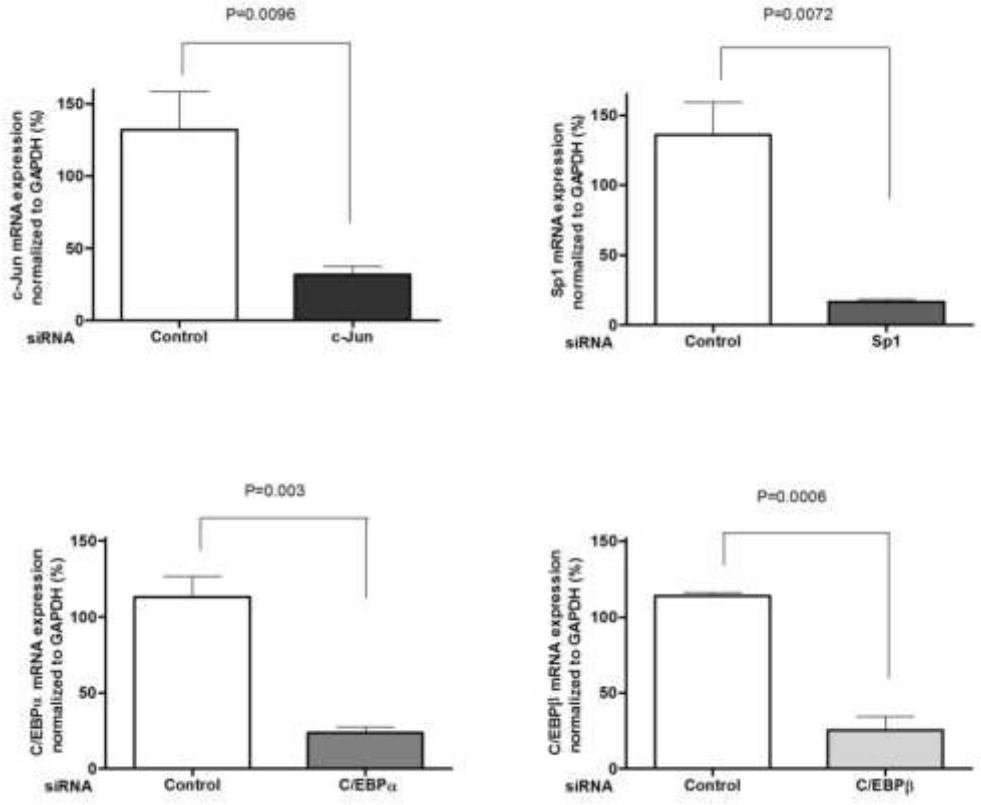


Figure 4.4 Identification of functional binding sites for AP-1, Sp1 and C/EBP. EMSA was performed with equal amounts of nuclear extracts from HCT116 and SW620 cells incubated with 5' biotin labeled MACC1 promoter oligonucleotides flanking binding sites specific for the three transcription factors: A) AP-1, B) Sp1, and C) C/EBPs. A reaction with 100x molar excess of unlabeled competitor sequence was also carried out indicating the specificity of the protein-DNA complexes. The reactions were analyzed by polyacrylamide gel electrophoresis. For supershift analysis, the nuclear extracts were incubated with biotin labeled oligonucleotides along with antibodies for c-Jun (A), Sp1 (B), C/EBPα and C/EBPβ (C). Binding of the antibodies resulted in disappearance of the protein-DNA binding complex. D) Equal amounts of HCT116 and SW620 chromatin were immunoprecipitated with antibodies for c-Jun, Sp1, C/EBPα and C/EBPβ and were quantified by agarose gel electrophoresis, using a primer set specific for the basal region (-426 to -18 bp). Non-immune IgG and input DNA served as negative and positive control respectively. Moreover, as a negative control, immunoprecipitated DNA was amplified by a primer set specific for an off target region (GAPDH) shown in the lower panel. [1]C/EBPα; [2]C/EBPβ.

4.1.5 AP-1, Sp1 and C/EBP regulate MACC1 mRNA and protein level

Until now, we established the binding of AP-1, Sp1 and C/EBP to the MACC1 promoter. Next, we wanted to understand the relevance of these transcription factors in governing MACC1 expression. So we employed RNAi strategy to knock down these transcription factors individually and assessed their effects on MACC1 expression at the mRNA as well as at the protein level. We first used qRT-PCR to confirm the target-specific predesigned siRNAs for their knockdown efficacy (Fig. 4.5 A). Based on this, we transfected these siRNAs into HCT116 cells and determined MACC1 mRNA expression by qRT-PCR. As shown in Fig. 4.5 B, siRNAs for c-Jun, Sp1, C/EBPα and C/EBPβ significantly reduced MACC1 mRNA levels by 2 fold ($P<0.01$), 1.5 fold ($P<0.05$), 1.8 fold ($P<0.01$) and 3.4 fold ($P<0.001$), respectively, as compared to a scrambled siRNA control sequence. Consistent with the mRNA levels, MACC1 protein expression was also down-regulated by siRNA treatment with the target specific siRNAs. The data clearly supports that these transcription factors, directly or indirectly, play an important role in determining MACC1 mRNA and protein expression and are indispensable for MACC1 regulation.

A



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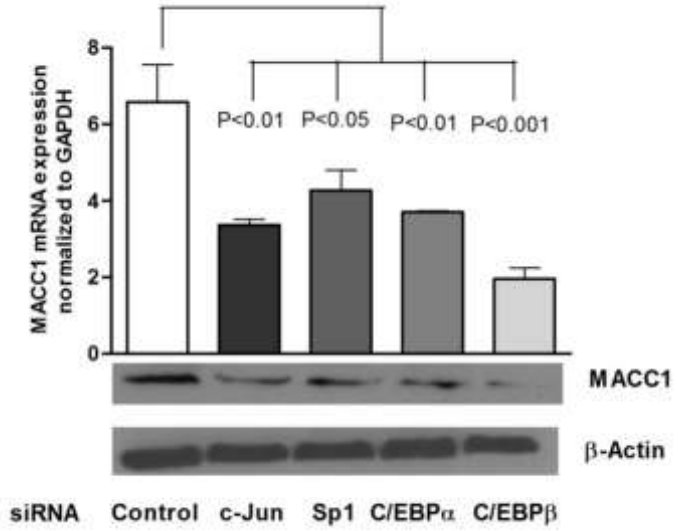


Figure 4.5 Implication of c-Jun, Sp1 and C/EBP in regulating MACC1 expression at the mRNA and the protein levels. siRNA knock-down was done to assess the effect of the transcription factors c-Jun, Sp1, C/EBPα and C/EBPβ on the MACC1 expression levels. HCT116 cells were transfected with siRNAs for each transcription factor. 48 h after transfection, total RNA was extracted, reverse transcribed and quantified by real time PCR. A) siRNA knock-down efficacy was validated for their respective target. Results are calculated as percentage of the untreated samples. B) Effect of siRNA on MACC1 expression. For MACC1 mRNA analysis, the data is normalized to GAPDH. Results are shown as means ± SEM of three independent experiments. For MACC1 protein analysis, cells transfected with siRNAs were harvested 48 h post transfection, and lysed in RIPA lysis buffer and the cell lysates were immunoblotted for MACC1 and β-Actin. A representative blot of three independent experiments is shown.

4.1.6 AP-1 and Sp1 knock-down abate MACC1-induced migration

MACC1 is a gene involved in CRC metastasis. MACC1-induced migration has already been described in HCT116 cells [119]. In order to ascertain the role of these transcription factors on MACC1-induced cell motility, we determined their effect on cell migration following knock down. HCT116 cells treated with siRNA for c-Jun or Sp1 showed significant inhibition of cell migration to 58% and 57% of control (both $P < 0.01$), respectively (Fig. 4.6 A). The knock-down of C/EBPα however, did not result in significant changes in migration ($P= 0.482$, data not shown) and was therefore not followed further together with C/EBPβ because of the opposing transcriptional effects driven by LAP and LIP, C/EBPβ isoforms. To address if the decreases in migration caused by AP-1 and Sp1 were at least partially driven via decrease in MACC1 expression, we designed rescue experiments employing HCT116 cells co-transfected with siRNA for c-Jun or Sp1 along with either pcDNA3.1 or pcDNA3.1/MACC1 plasmid (Fig. 4.6 B). Overexpression of ectopic MACC1 rescued the effect of transcription factor knock-down-induced low migratory capability. In summary, AP-1 and Sp1 siRNA treatment restricted the migratory capabilities of HCT116 cells and the ectopic expression of

MACC1 was able to overcome AP-1 and Sp1-mediated migratory inhibition demonstrating that these effects are MACC1-specific.

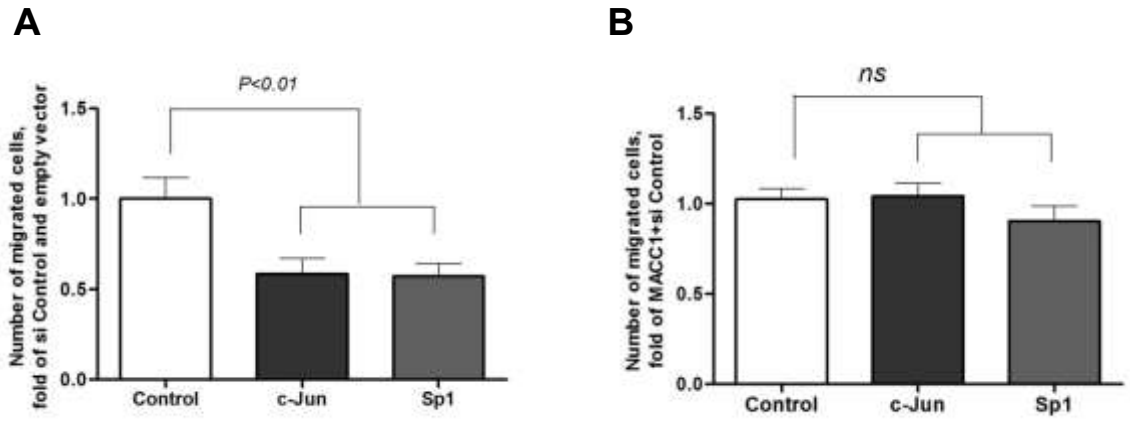


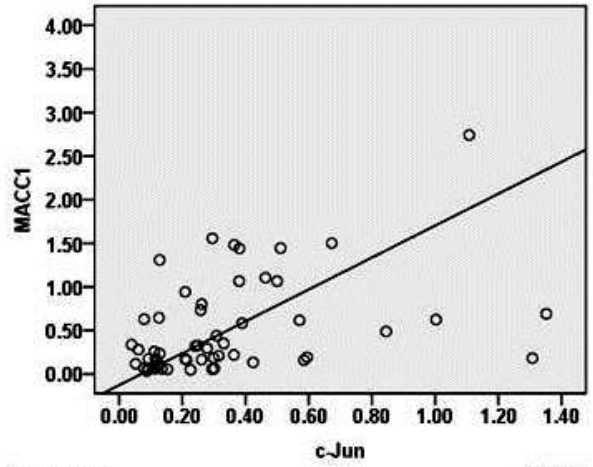
Figure 4.6 Migratory abilities of AP-1 and Sp1 siRNA treated cells. A) HCT116 cells were co-transfected with pcDNA3.1/empty plasmid and si control or siRNA for c-Jun or Sp1 for 48 h. Cell migration was counted using Boyden chamber assay and expressed as fold over si control treated cells. B) For rescue experiment, HCT116 cells were co-transfected with pcDNA3.1/MACC1 plasmid and siRNA for c-Jun or Sp1 for 48 h and cell migration was counted. Each migration assay was performed in triplicates and average of two independent experiments was plotted in the graph. ns represent data is not significant.

4.1.7 AP-1 and Sp1 levels correlate with the MACC1 expression in human colorectal tumors

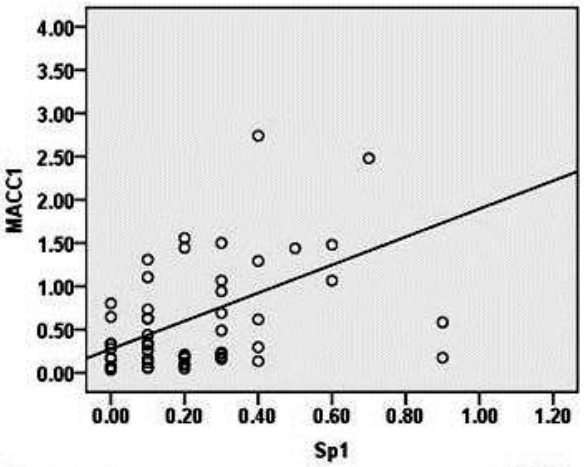
To evaluate the correlation of AP-1 and Sp1 with MACC1 in clinical specimens of CRC, we used RNA from patient samples described in our previous study [108]. All patients underwent surgical R0 resection and showed no distant metastasis at the time of surgery and neither had they any history of familial CRC, or a second tumor of the same entity. We performed qRT-PCR for MACC1, c-Jun and Sp1. By using Spearman correlation method, we found a positive correlations between MACC1 and c-Jun (r = 0.5897) as well as between MACC1 and Sp1 (r = 0.5153) indicating positive statistical dependence of two variables with each other (Fig. 4.7 A). We also looked for the biological dependence of AP-1 and Sp1 on MACC1. Thus, we classified the patients with low MACC1 expression and high MACC1 expression using the median cut-off values. We then determined c-Jun and Sp1 levels in the two cohorts. Remarkably, we found that the tumors with low MACC1 expression also showed low expression of both c-Jun and Sp1, whereas high MACC1 expressers showed elevated level of c-Jun and Sp1 (P = 0.0007 and P = 0.02, respectively), (Fig. 4.7B). Furthermore, patients who developed metachronous metastases were shown to have significantly more c-Jun and Sp1 levels compared with those that did not develop metastasis over 12 years (P =

0.01 and P = 0.001, respectively), (Fig. 4.7 C). Our data indicates that in the clinical setting, Ap-1 and Sp1 might be important players for regulating MACC1 and MACC1-induced metastasis.

A

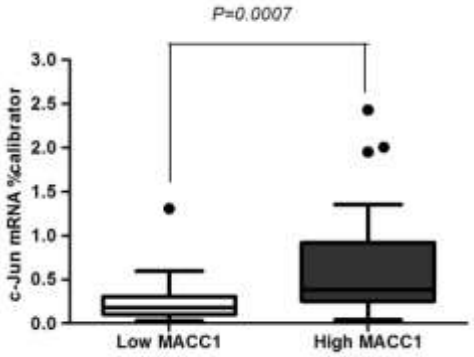


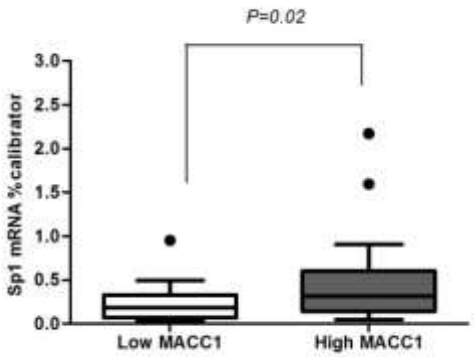
Spearman r	0.5897
95% confidence interval	0.3884 to 0.7373
P value (two-tailed)	< 0.0001
P value summary	***



Spearman r	0.5153
95% confidence interval	0.2914 to 0.6856
P value (two-tailed)	< 0.0001
P value summary	***

B





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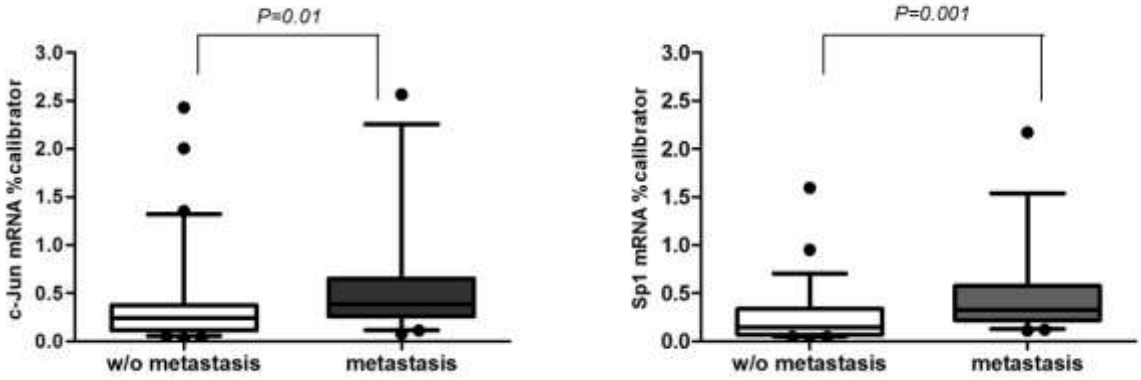


Figure 4.7 Correlation of MACC1 levels with AP-1 as well as Sp1 levels in colorectal tumors. qRT-PCR was performed on cDNA obtained from colorectal primary tumors (n = 60, stages 1-3) with 23 patients that developed distant metastases in the following twelve years. A) Spearman correlation coefficient analysis for MACC1 and c-Jun as well as for MACC1 and Sp1 was done with non-parametric data obtained from qRT-PCR results and correlation coefficient 'r' was calculated. B) Box plot analyses comparing c-Jun as well as Sp1 levels among the low MACC1 and high MACC1-expressing tumors. C) Box plot analyses comparing c-Jun as well as Sp1 levels among those tumors that did not metastasize (w/o metastasis) or that developed metastases metachronously. All qRT-PCR results are normalized with GAPDH.

4.2 GIPC1 as a novel potential transcription factor regulating MACC1

4.2.1 GIPC1 silencing down regulates MACC1 expression

GIPC1, a cytoplasmic scaffold protein with a PDZ domain, binds to numerous proteins and is involved in multiple biological processes, including cell migration. Our aim was to evaluate whether GIPC1 reduces migration by altering the levels of MACC1. Therefore, we made stable SW620 cell line with knocked-down GIPC1 (SW620-shGIPC1) using shRNA constructs. shRNA scrambled sequence was used as a control. We isolated RNA from these two cell lines and measured MACC1 mRNA expression by qRT-PCR (Fig. 4.8). We observed a 50% decrease in MACC1 expression in SW620-shGIPC1 cells as compared to SW620-shControl cells suggesting the association of GIPC1 with MACC1.

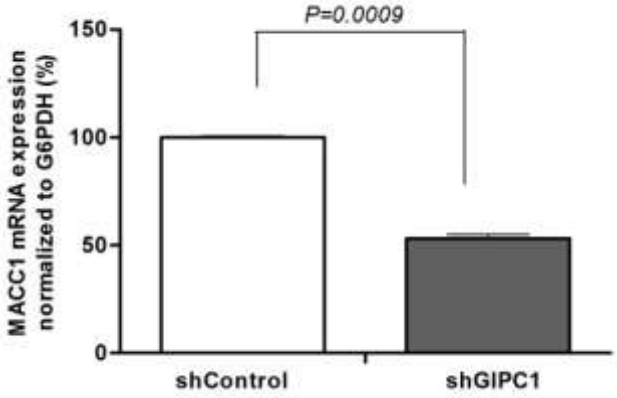


Figure 4.8 Effect of GIPC1 knock-down on MACC1 expression. shRNA transfection was done to knock down GIPC1 in SW620 cells. Stable clones were picked and validated. Total RNA was isolated from these cells, reverse transcribed and quantified using real time PCR. The data is normalized for G6PDH. Results are shown as means ± SEM of two independent experiments performed in duplicate.

4.2.2 GIPC1 regulates the MACC1 promoter

We observed that knock down of GIPC1 leads to reduced MACC1 expression. We then evaluated whether this effect mediated by GIPC1, is through regulating the MACC1 promoter activity. We transfected SW620-shControl and SW620-shGIPC1 cells with the full length MACC1-promoter luciferase construct (MACC1p-992) or empty vector alone. 24 h after transfection, we measured the luciferase activity. We found 48% decrease in the MACC1 activity in GIPC1 knock down cells (Fig. 4.9 A) indicating the possible interaction of GIPC1 with the MACC1 promoter. We were inquisitive about the region where it binds on the MACC1 promoter. So we transfected our previously described 5' truncated MACC1 promoter deletion constructs in SW620-shControl and SW620-shGIPC1 cells and calculated the reduction in the MACC1 promoter activity. We detected no significant reduction in MACC1 promoter activity among different promoter constructs (Fig. 4.9 B) suggesting that the

smallest MACC1 promoter fragment (MACC1p-133) is sufficient enough for MACC1- GIPC1 interaction.

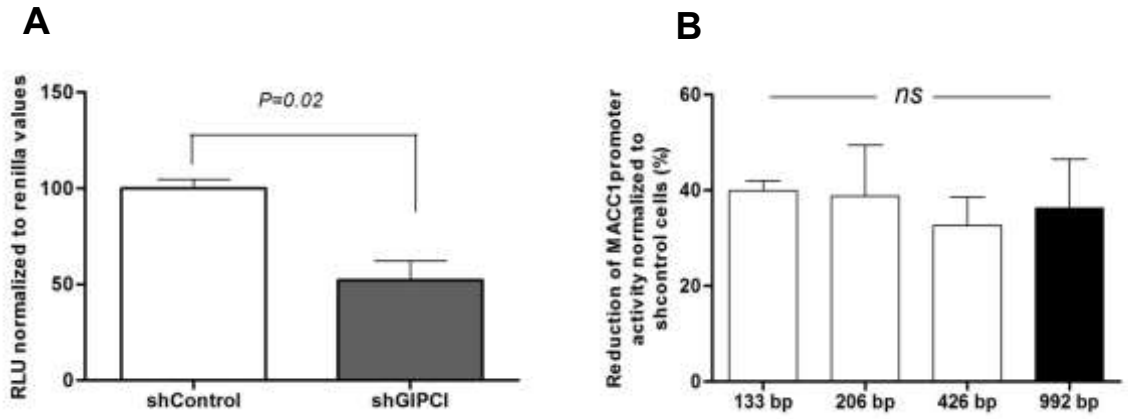


Figure 4.9 Effect of GIPC1 on the MACC1 promoter activity. A) MACC1 promoter luciferase reporter construct was co-transfected along with pGL4.74 Renilla plasmid in SW620-shcontrol and SW620-shGIPC1 cells. After 24 h of transfection, luciferase activity was measured and was normalized to Renilla luciferase activity to correct for the variation in transfection efficiencies. Luciferase activity from the SW620-shcontrol cells was set to 100%. B) Fragments of the MACC1 promoter with deletions at the 5' end and possessing a common 3' end were inserted into pGL4.17 and were transfected into SW620-shGIPC1 and SW620-shControl cells. Luciferase activity was normalized to Renilla values and expressed as percentage reduction of luciferase activity in SW620-shGIPC1 as compared with SW620-shcontrol cells.

4.2.3 GIPC1 physically interacts with the MACC1 promoter

To demonstrate the physical binding of GIPC1 to the MACC1 promoter, we performed an EMSA assay. We designed biotin labeled oligonucleotides corresponding to the first 60 bp from TSS in the MACC1 promoter. On incubating 5' labelled MACC1 promoter fragment with nuclear extract from SW620 cell line, we observed DNA-protein complex formation which disappeared on adding 100 fold molar excess of unlabeled promoter fragment. The specificity of the complex was determined by addition of an antibody for GIPC1 which led to a decrease in the intensity of the specific shift (Fig. 4.10 A).

Additionally, we analyzed the specific physical interaction of GIPC1 with the MACC1 promoter by ChIP assay. A clear strong band was seen on amplification of immune precipitated chromatin by GIPC1 antibody with the primers for the MACC1 promoter (Fig. 4.10 B). Taken together, the results from both ChIP and EMSA analysis confirmed the physical binding of GIPC1 to the MACC1 promoter.

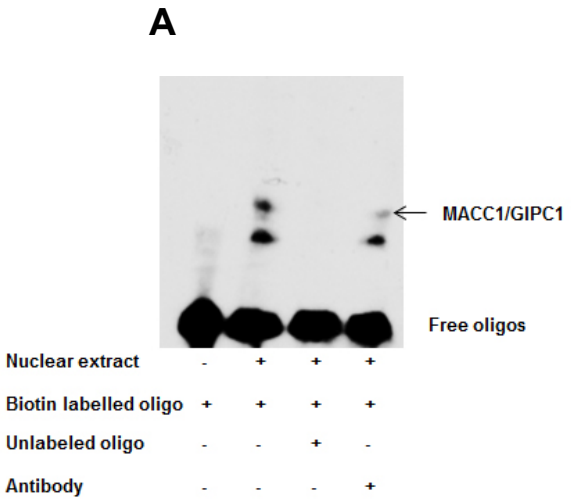


Figure 4.10 GIPC1 binds to the first 60 bp of the MACC1 promoter. A) 5' end biotin labeled oligonucleotide corresponding to MACC1 promoter were incubated alone as well as with nuclear extracts from SW620 cells along with 100x molar excess of unlabeled competitor sequence to indicate specificity of the protein-DNA complexes or with the antibody specific for GIPC1. The reactions were analyzed by polyacrylamide gel electrophoresis. N.S. indicates non-specific complex. B) SW620 chromatin was immunoprecipitated with antibodies for GIPC1 antibody and quantified by agarose gel electrophoresis, using a primer set specific for the MACC1 promoter and GAPDH. Non-immune IgG and input DNA without any immunoprecipitation with antibody served as negative and positive controls respectively.

4.3 Identification of small molecule transcriptional inhibitors of MACC1

4.3.1 High-throughput screening led to the identification of small molecule transcriptional inhibitors of MACC1

High-throughput screening (HTS) is a state-of-the-art technique in the field of drug discovery. Thus, in collaboration with Dr. Jens Von Kries (MDC, Berlin), we carried out a HTS using a Chembionet library of more than 30,000 compounds to identify small molecule inhibitors targeting the MACC1 gene promoter. The design of the screening process and downstream deconvolution strategy has been represented diagrammatically, followed by the outcome of the screen. Information on the novel inhibitors discovered and their behavior in dose-dependence assays has also been provided, leading ultimately to the identification of Rottlerin and Lovastatin as the MACC1 transcriptional inhibitors of choice for further study.

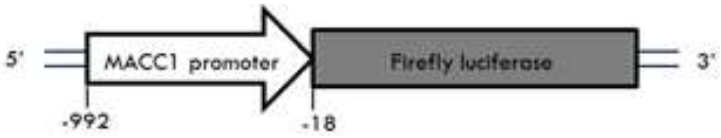
4.3.1.1 Pilot scale study led to design and optimization of the screening strategy

We selected a cell based, luciferase-reporter assay to identify transcriptional inhibitors of MACC1. As described earlier, MACC1 promoter was cloned upstream of the luciferase gene in the pGL4.17 plasmid. (Fig.4.11 A).Herein, we transfected HCT116 cells with this construct, to generate a cell line stably expressing MACC1 promoter integrated with the luciferase gene. The selection of stable cell line was based on G418 resistance carried over by the pGL4.17 plasmid. As a negative control, pGL4.17 plasmid without any promoter before the luciferase gene was used.

In order to establish a cell-based assay for HTS to identify small molecule MACC1 transcriptional inhibitors, a large number of assay parameters were needed to be optimized first in a pilot scale done manually, followed by an assay transfer to a robotic system. These parameters included different 384-well plate formats (white opaque or clear bottom), DMSO concentrations, cell number, integration time for measuring luminescence and an optimal z factor (accounting for the suitability of experimental design, working positive and negative controls to be used for HTS). After repeating the assay with each of these variable parameters mentioned above, we finally optimized the screening for 5000 cells per plate in a white opaque 384-well plate with a moderate to good z factor ranging from 0.4 to 0.6 for the final screening. Further we selected integration time of 500 ms for luciferase measurement. Fig. 4.11 B depict optimization of DMSO concentration to be used for drug treatment in the HTS after finalizing the cell number and integration time of 5000 cells and 500 ms respectively. As shown in table, increase in DMSO concentration from 0.25% to 0.5% led to a slight increase in the promoter activity and therefore we restricted ourselves to a minimum possible concentration of 0.25%. Then we ran a test plate (assay transfer, Fig. 4.11C) with

DMSO treated empty vector cells (negative control, depicted in red) in the first column and DMSO treated MACC1 promoter cells (positive control, depicted in blue) in the second column as shown in Fig. 4.11 C, and performed drug treatment with few inhibitors on rest of the plate having MACC1 promoter cells followed by luciferase assay after 24 h to measure MACC1 promoter activity. Our test plate worked efficiently and potential hits were found as indicated by reddish-brown squares.

A



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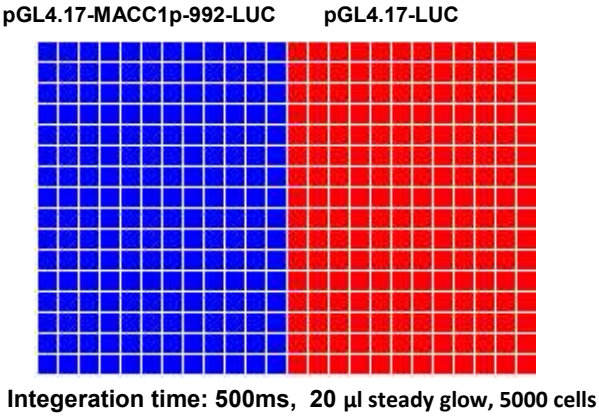
B



Integeration time: 500ms, 20 µl steady glow, 5000 cells

No DMSO	
Mvpos	1832.94
Mvneg	635.04
Sdpos	177.23
Sdneg	41.01
Z factor	0.45

0.25% DMSO	
Mvpos	2590.65
Mvneg	352.6
Sdpos	347.13
Sdneg	47.14
Z factor	0.47

0.5% DMSO	
Mvpos	3210.83
Mvneg	352.81
Sdpos	415.22
Sdneg	48.13
Z factor	0.51

C

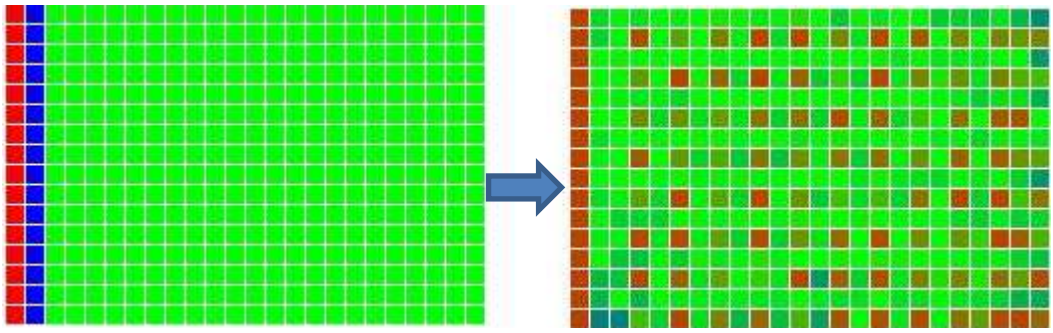


Figure 4.11 Design and optimization for the luciferase based screening process.
A) Schematic representation of the reporter system used in the high-throughput screening. The expression of firefly luciferase gene was regulated by the MACC1 promoter (-18 to -992 bp upstream of the TSS). B) Three 384-well plates were seeded with MACC1p-992-LUC cells (in blue) and LUC cell without any promoter (in red). Cells were then treated with or without DMSO (0.25% and 0.5%) for 24 h and luciferase activity were measured as shown in the table. Mvpos represent mean value of MACC1 promoter cells, Mvneg represents mean value of empty vector cells, Sd represents standard deviation. C) Assay transfer with selected inhibitors as a test run wherein, red column contains pGL4.17 cells without any promoter treated with DMSO, blue column represents MACC1p-992-LUC cells treated with DMSO and rest of the green columns represent MACC1p-992-LUC cells treated with test compounds (left plate). Right plate represents MACC1 promoter driven luciferase activity in a scale from orange to green, orange being the minimum and green representing basal luciferase value and blue representing maximum luciferase activity.

4.3.1.2 Identification of 97 novel small molecules as MACC1 transcriptional inhibitors

For carrying out HTS, HCT116-MACC1p-992-LUC CRC cells stably expressing a human MACC1 promoter-driven luciferase reporter gene construct were used to screen the Chembionet library of more than 30,000 compounds which includes 1280 compounds from Sigma LOPAC library for identifying potential MACC1 inhibitors. In the primary screen, using 5 µM concentration of each compound, we identified 542 compounds that inhibited MACC1 promoter-driven luciferase expression by greater than 50% as compared to solvent treated control cells (schematic representation shown in Fig. 4.12 A). These 542 compounds were then subjected to a selectivity screen with HCT116-CMVp-Luc cells, wherein luciferase reporter gene is driven by CMV promoter instead of the MACC1 promoter. The compounds which inhibited luciferase expression with CMV driven cells by greater than 75% were considered as non-specific luciferase inhibitors or cytotoxic compounds leading to a decrease in the promoter activity. Out of 542 compounds, 445 compounds were found to inhibit CMV promoter driven luciferase expression and thus, were eliminated. Therefore, we were left with 97 effective compounds, which included 7 pharmacologically active compounds from LOPAC library and 90 novel chemical compounds. To confirm the inhibitory potential of the remaining 97 effective compounds and to establish the concentration response curves, the 97 compounds were titrated using 10 two fold serial dilutions starting with the highest concentration of 50 µM and luciferase assay was performed. On the basis of curve properties (Hill coefficient, Chi squared values and IC_{50} values), solubility and purity of

the drug, selectivity screen comparison, information on known biological targets, known functions and Z score, two out of seven pharmacologically active hits, Rottlerin and Mevastatin, emerged as strongest candidates for MACC1 transcriptional inhibition.

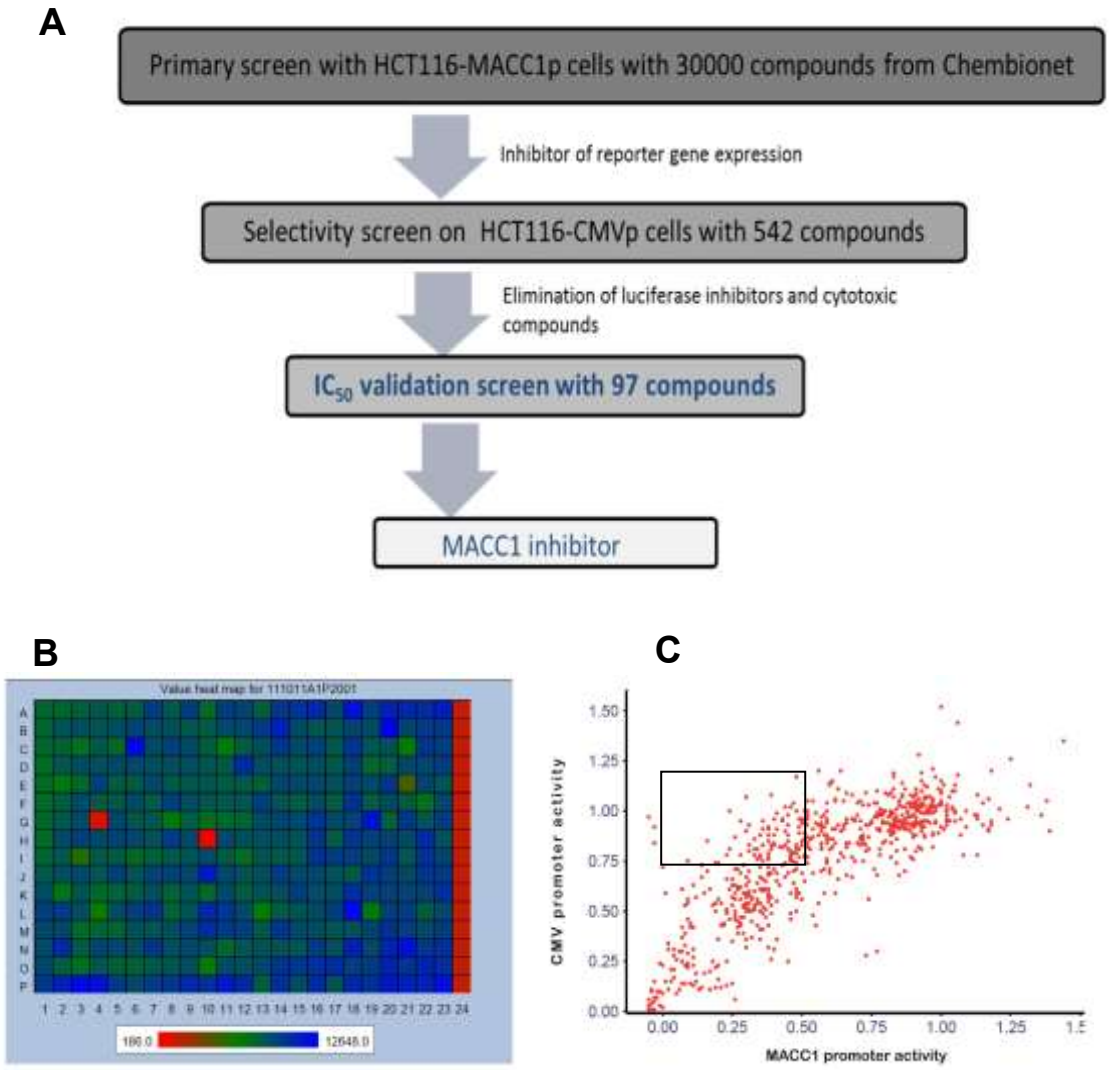


Figure 4.12 Schematic representation of high-throughput screening. A) Diagrammatic representation of the screening process. MACC1 promoter expressing cells were screened with chembionet compounds in a 384-well plate format. Screening consisted of primary screening followed by a selectivity screen and finally concentration-response assays with the potential hits. B) Heat map of a representative plate from a primary screen where red color represent minimum promoter activity and blue color represent maximum promoter activity. C) A scatter plot representing selectivity screen with 542 compounds. Cut off window of 50% was set for MACC1 promoter activity and 75% for CMV promoter activity and 97 compounds were then selected which decreased MACC1 promoter activity by more than 50% and CMV promoter to not more than 75%.

4.3.1.3 Rottlerin and Mevastatin inhibit MACC1 promoter activity

In concentration response assay for measuring MACC1 promoter activity, Rottlerin showed inhibition of luciferase activity from concentration of 0.78 µM onwards and Mevastatin

showed inhibition of luciferase activity at 1.56 µM and higher concentrations. We next wanted to evaluate whether this decrease in promoter activity is contributed by decrease in cell number, therefore, we performed a MTT assay to assess the effects of these drugs on cell viability. Rottlerin reduced cell viability at 6.25 µM and higher concentrations whereas Mevastatin reduced cell viability at 12.5 µM and higher concentrations (Fig. 4.13). Thus, the concentration-response assays confirmed that Rottlerin and Mevastatin inhibit MACC1 promoter-driven reporter gene expression at non-cytotoxic concentrations and thus, we selected them for further studies as potential small molecule MACC1 transcriptional inhibitors.

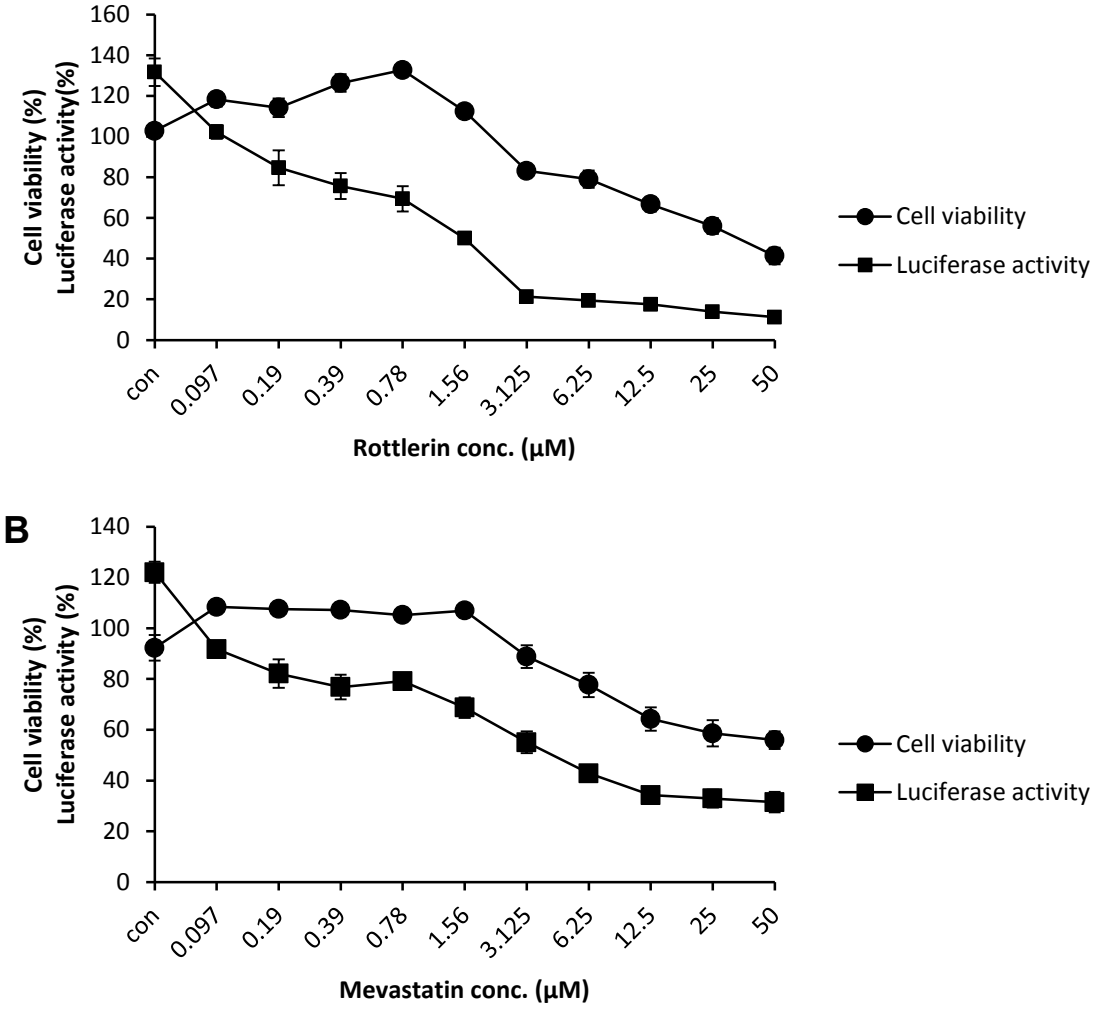


Figure 4.13 Identification of Rottlerin and Lovastatin as MACC1 transcriptional inhibitors via high-throughput screening. HCT116-MACC1p-Luc cells were treated with 10 two-fold serial dilutions of Rottlerin (A) and Mevastatin (B) for 24 h, starting with 50 µM concentration. Luciferase activity was determined using steady glow luciferase reagent and normalized to untreated cells. Cell viability was measured independently using MTT assay. Results are shown as means ± SEM of two independent experiments performed in triplicates.

4.3.2 Small molecule inhibitors restrict MACC1 expression

4.3.2.1 Rottlerin and Mevastatin inhibit MACC1 expression in a concentration dependent manner

To determine the ability of the small molecule inhibitors to reduce endogenous MACC1 expression, HCT116 cells were treated with increasing concentrations of Rottlerin or Mevastatin for 24 h. Rottlerin treatment reduced the MACC1 mRNA level in a concentration-dependent manner (Fig. 4.14 A). At a concentration of 2.5 µM, Rottlerin showed more than 60% reduction in the endogenous MACC1 mRNA and MACC1 protein level compared to the solvent-treated control.

Similar to the effects seen for Rottlerin, increasing concentrations of Mevastatin also inhibited MACC1 mRNA expression in a concentration-dependent manner (Fig. 4.14 B). A small reduction by 26% in the MACC1 mRNA level was seen when HCT116 cells were treated with 2.5 µM. Treatment with 5 µM Mevastatin significantly restricted the MACC1 mRNA level to less than 50% of the solvent-treated control. Additionally, the reduction in MACC1 protein expression was clearly observed upon treatment with Mevastatin at 5 µM or higher concentrations.

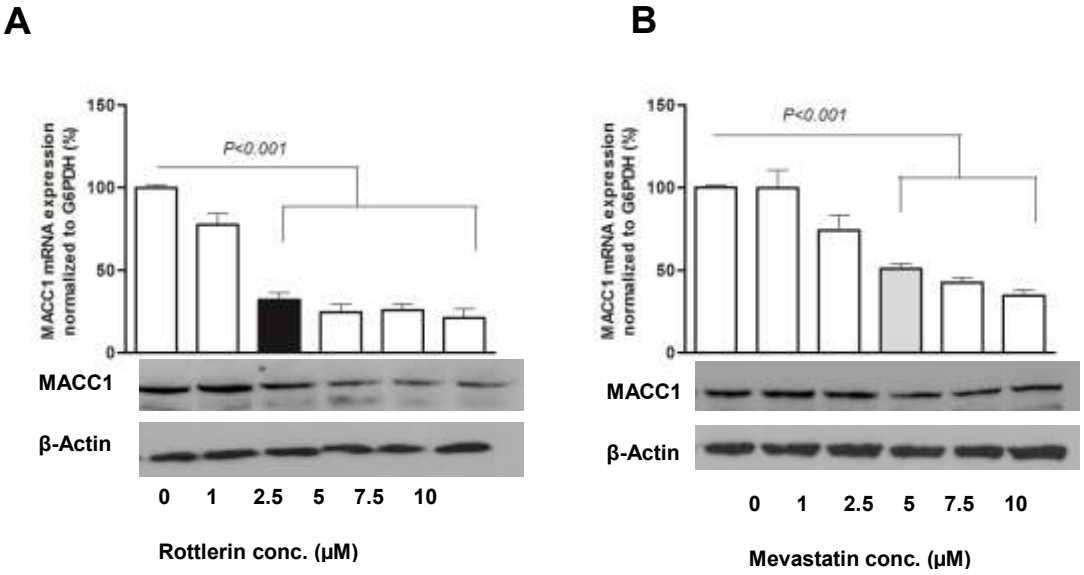


Figure 4.14 Effect of Rottlerin and Mevastatin on MACC1 expression. HCT116 cells were treated with increasing concentrations of Rottlerin (A) or Mevastatin (B) for 24 h and MACC1 mRNA and protein expression was determined by qRT-PCR and Western blot analysis, respectively. Effective MACC1 inhibitory concentration of Rottlerin is shown in black bar and for Mevastatin is shown in grey bar. Data represent means ± SEM from three independent experiments.

4.3.2.2 Lovastatin inhibits MACC1 expression in a concentration dependent manner

Due to severe toxicity profile in clinical trials, Mevastatin was never introduced in the market. The first class of statins that entered the market and got FDA approval was Lovastatin [159]. Therefore, we wanted to know whether Lovastatin (a FDA approved analogue of Mevastatin)

has the same effect on MACC1 as Mevastatin. We performed a concentration response curve using 10 two-fold serial dilutions of Lovastatin as performed earlier for other compounds and analyzed its effect on cell viability and luciferase activity in HCT116-MACC1p-Luc cells. Lovastatin inhibited luciferase activity at 0.39 µM and higher concentrations and reduced viability at 3.125 µM and higher concentrations (Fig. 4.15 A).
Furthermore, to study the effect of Lovastatin on MACC1 expression, we treated HCT116 cells with increasing concentrations of the inhibitor as done previously. Similar to Mevastatin, treatment with 5 µM of Lovastatin significantly reduced MACC1 mRNA levels by 62% as compared to solvent-treated control (Fig. 4.15 B). Additionally, treatment with Lovastatin induced a clear decrease in MACC1 protein level as demonstrated by western blotting. Altogether, these results demonstrate that Lovastatin and Mevastatin have similar potential as MACC1 inhibitors. Therefore, considering that, Lovastatin is accepted for therapeutic use in contrast to Mevastatin, we decided to work with Lovastatin from this point on.

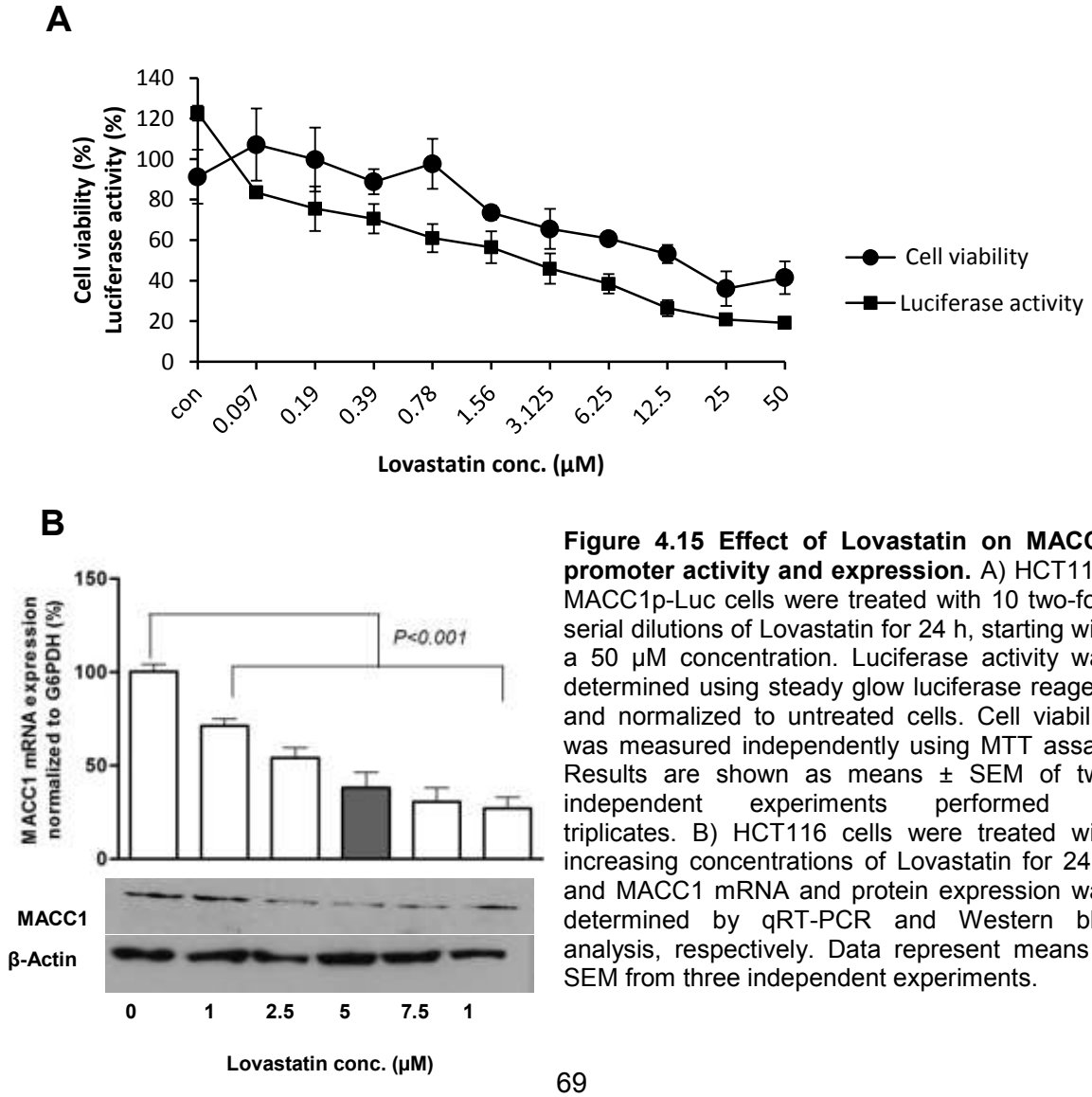


Figure 4.15 Effect of Lovastatin on MACC1 promoter activity and expression. A) HCT116-MACC1p-Luc cells were treated with 10 two-fold serial dilutions of Lovastatin for 24 h, starting with a 50 µM concentration. Luciferase activity was determined using steady glow luciferase reagent and normalized to untreated cells. Cell viability was measured independently using MTT assay. Results are shown as means ± SEM of two independent experiments performed in triplicates. B) HCT116 cells were treated with increasing concentrations of Lovastatin for 24 h and MACC1 mRNA and protein expression was determined by qRT-PCR and Western blot analysis, respectively. Data represent means ± SEM from three independent experiments.

Our results (Fig. 4.14 and Fig. 4.15) demonstrate that Rottlerin and Lovastatin can reduce MACC1 mRNA and protein levels in a dose dependent manner. For further experiments, a drug concentration with a minimal effect on cell viability and a maximum effect on MACC1 expression inhibition had to be selected. Therefore, a concentration of 2.5 µM for Rottlerin and 5 µM for Lovastatin was chosen for all further experiments.

4.3.2.3 Rottlerin and Lovastatin inhibit MACC1 expression in a time dependent manner

After evaluating the effective concentrations of Rottlerin and Lovastatin for inhibiting MACC1 expression, the time dependent effect of these inhibitors on the MACC1 expression was analyzed. After 12 h exposure of HCT116 cells to a single dose of 2.5 µM Rottlerin, the MACC1 mRNA was significantly reduced to less than 50% of the solvent-treated control (Fig. 4.16 A). After 18 h, the MACC1 mRNA expression was reduced to 26% of the solvent treated cells that persisted until 62 h beyond which the cells could not be harvested due to the depletion of nutrients in the media. Thus, treatment of HCT116 cells with a single dose of 2.5 µM of Rottlerin was sufficient to reduce the MACC1 mRNA to less than 30% of solvent-treated control cells for at least 62 h. In agreement with the mRNA profile, reduction in the MACC1 protein was also detected 12 h post treatment with 2.5 µM of Rottlerin which stayed until 62 h following a single dose.

Similarly, on analyzing the kinetics underlying the Lovastatin-mediated inhibition of the MACC1 expression, we found that a single dose of Lovastatin reduced the MACC1 expression in a time dependent manner. After 12 h treatment of HCT116 cells with 5 µM of Lovastatin, the MACC1 mRNA level was already significantly reduced to 56% of the solvent-treated control cells, which further decreased to 16% at 48 h. This was followed by a very slight increase at 62 h (Fig. 4.16 B). Reduction in MACC1 protein was observed 24 h post treatment which gradually decreased until 48 h followed by a slight increase at 62 h.

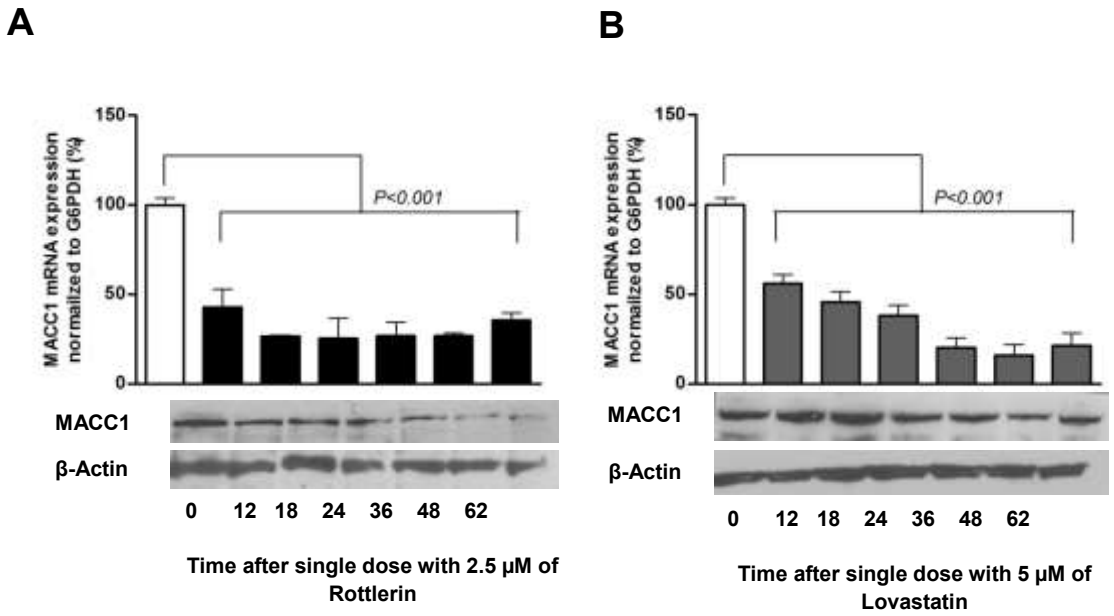
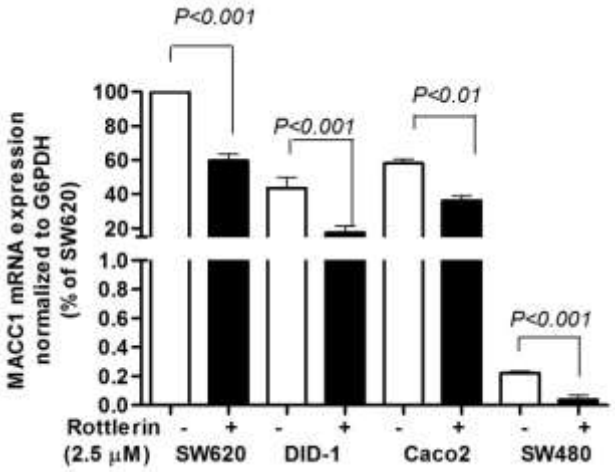


Figure 4.16 Time dependent kinetics of Rottlerin and Lovastatin on MACC1 expression. HCT116 cells were treated with a single dose of 2.5 µM Rottlerin or 5 µM Lovastatin for the time indicated. MACC1 mRNA and protein was analyzed by qRT-PCR and Western blot, respectively. Data represent mean ± SEM from three independent experiments.

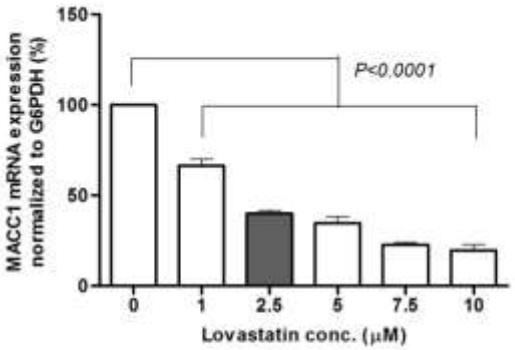
4.3.2.4 Rottlerin and Lovastatin restrict MACC1 expression in a panel of human CRC cells

Next, we analyzed the effects of these inhibitors on the MACC1 expression in different CRC cell lines. Upon treatment of SW620, DLD-1, Caco2 and SW480 cells with 2.5 µM Rottlerin, MACC1 mRNA levels were reduced to 60%, 40%, 62% and 18% of the solvent treated control (Fig. 4.17 A). Similarly, Lovastatin was capable of restricting MACC1 expression in various human CRC cells but, unlike Rottlerin, the significantly effective concentration was cell line dependent. In the case of SW48 cells, even 2.5 µM of Lovastatin was sufficient to reduce MACC1 mRNA to 40%. In the case of DLD-1 cells, 30 µM of Lovastatin was required to observe a 50% decrease in MACC1 mRNA levels whereas for SW620 cells, a higher concentration of 50 µM was required to restrict MACC1 expression to 55% (Fig. 17 B, C, D). We also performed a MTT assay to determine the toxicity profile of higher concentrations of Lovastatin on these cells. Our data (Fig. 4.15 E, F) demonstrates different sensitivity profiles of these CRC cell lines towards Lovastatin treatment and in all the cell lines, MACC1 expression was inhibited at non-toxic concentrations.

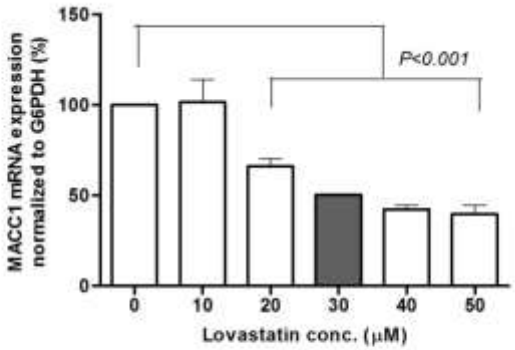
A


P<0.001
P<0.001
P<0.01
P<0.001
MACC1 mRNA expression normalized to G6PDH (% of SW620)
100
80
60
40
20
1.0
0.8
0.6
0.4
0.2
0.0
Rottlerin - + - + - + - +
(2.5 μM) SW620 DID-1 Caco2 SW480

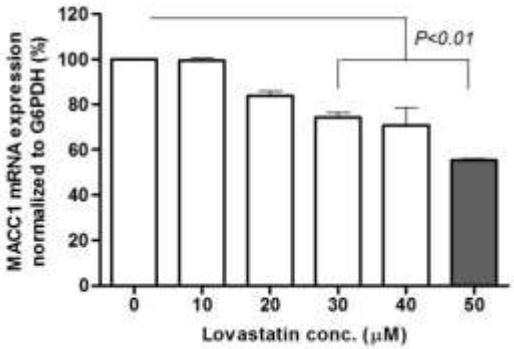
B


P<0.0001
MACC1 mRNA expression normalized to G6PDH (%)
150
100
50
0
0 1 2.5 5 7.5 10
Lovastatin conc. (μM)

C


P<0.001
MACC1 mRNA expression normalized to G6PDH (%)
150
100
50
0
0 10 20 30 40 50
Lovastatin conc. (μM)

D


P<0.01
MACC1 mRNA expression normalized to G6PDH (%)
120
100
80
60
40
20
0
0 10 20 30 40 50
Lovastatin conc. (μM)

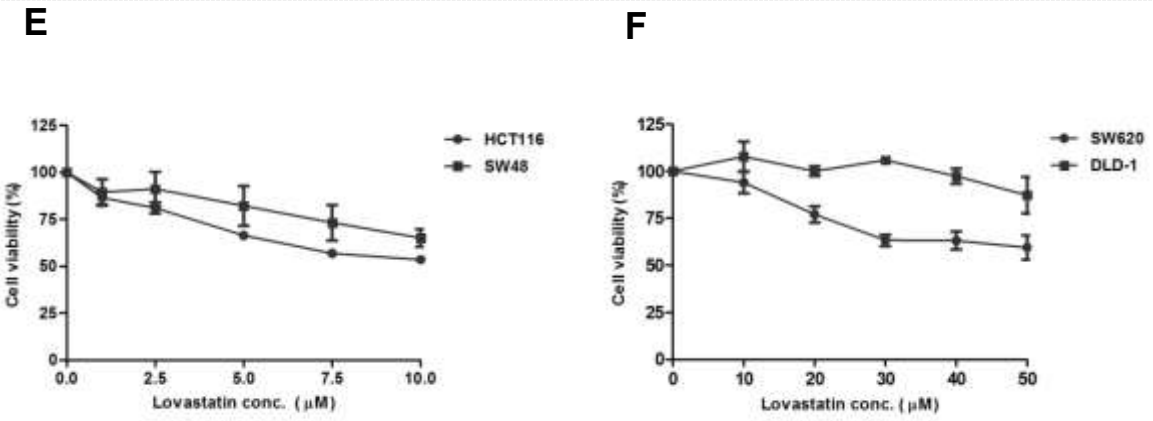


Figure 4.17 Effect of Rottlerin and Lovastatin on MACC1 expression in various CRC cell lines. A) CRC cells were treated with 2.5 µM Rottlerin for 24 h. MACC1 mRNA expression was analyzed by qRT-PCR and results were normalized with G6PDH and expressed as percentage of solvent treated SW620 cells. Increasing dose of Lovastatin treatment was carried out in (B) SW48 (C) DLD-1 (D) SW620 cells and MACC1 mRNA was quantified using qRT-PCR. Data represent mean ± SEM from three independent experiments. E) HCT116 and SW48 cells were treated with increasing concentrations of Lovastatin for 24 h and then MTT assay was performed. F) SW620 and DLD-1 cells were treated with a higher concentration range of Lovastatin for 24 h and MTT was performed. Percentage viability of treated cells was calculated over solvent treated control cells. Data represent means ± SEM (n≥2).

4.3.3 Small molecule inhibitors do not affect exogenous MACC1 expression

To demonstrate that Rottlerin and Lovastatin are transcriptional inhibitors of MACC1, we hypothesized that exogenous overexpression of MACC1 governed by a promoter other than its own MACC1 promoter should be resistant to the inhibitory effects of our drugs. Therefore, HCT116 cells were transiently transfected to express CMV-promoter-driven MACC1 cDNA. As result, HCT116/MACC1 cells had at least 10 times increased MACC1 mRNA level compared to HCT116/vector cells. The MACC1 protein level too was clearly increased in these cells as compared to HCT116/vector cells.

Treatment of HCT116/vector cells with 2.5 µM Rottlerin (Fig. 4.18 A) or 5 µM Lovastatin (Fig. 4.18 B) reduced the MACC1 mRNA level to more than 50% of the solvent-treated control, which was similar to the effect observed in HCT116 cells. In contrast, treatment of HCT116/MACC1 cells with the same concentration of Rottlerin or Lovastatin did not result in a significant change in the MACC1 mRNA. Similar to the effects observed at mRNA levels, MACC1 protein expression in the HCT116/vector cells was decreased when cells were treated with 2.5 µM Rottlerin or 5 µM Lovastatin but remained unchanged in the drug treated HCT116/MACC1 cells.

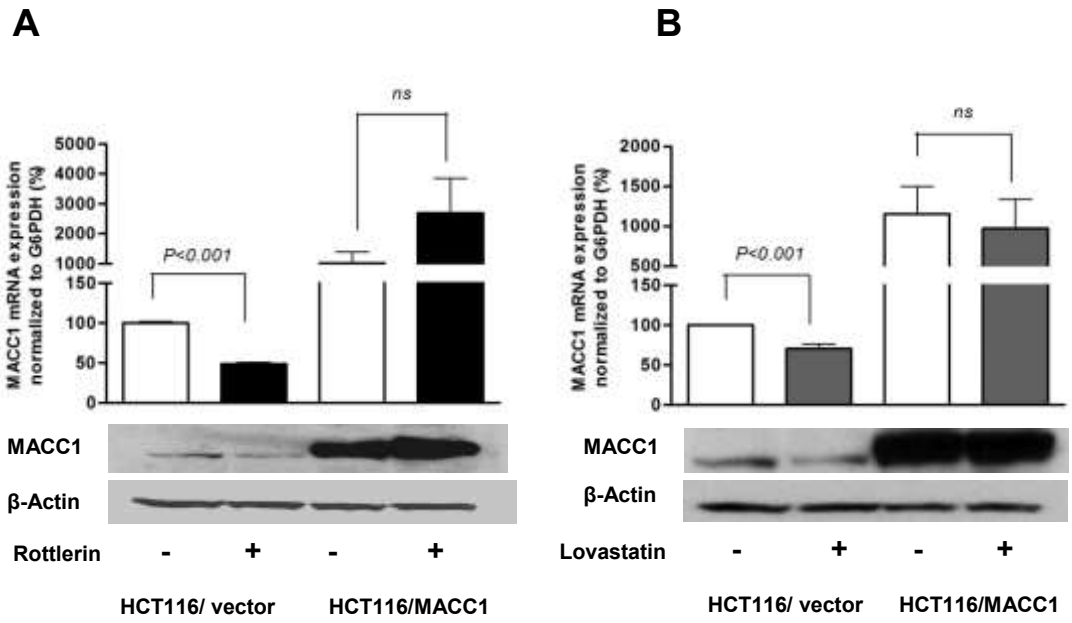


Figure 4.18 Effect of Rottlerin and Lovastatin on HCT116/vector and HCT116/MACC1 cells. HCT116 cells were transiently transfected to express CMV-promoter-driven MACC1 cDNA (HCT116/MACC1) or the empty vector (HCT116/vector) as control. Cells were treated with 2.5 µM Rottlerin (A) or 5 µM Lovastatin (B) for 24 h and MACC1 mRNA and protein levels were analyzed by qRT-PCR and Western blot, respectively. Data represent means ± SEM (n>3).

4.3.4 Small molecule inhibitors decrease cell proliferation and migration

4.3.4.1 Rottlerin and Lovastatin inhibit cell proliferation

One of the hallmark of cancer is enhanced proliferation rate of the cancer cells [160]. Therefore, for the identification of any anti-cancer drug, it is important to characterize its effect on proliferation. Moreover, MACC1 has been shown to be responsible in conferring properties contributing to metastasis, such as increased cell motility and proliferation, which are one of the first cellular processes that trigger metastasis formation [108]. Thus inhibition of MACC1 should lead to reduction in cell motility and proliferation. In order to evaluate that, we performed MTT assay to analyze anchorage-dependent proliferation of HCT116 cells upon treatment with small molecule MACC1 inhibitors. Treatment of HCT116 cells with either 2.5 µM of Rottlerin or 5 µM Lovastatin daily resulted in a significantly reduced cell proliferation after 48 h (Fig. 4.19). On day 3, Lovastatin only partially inhibited proliferation of HCT116 cells whereas Rottlerin completely attenuated the growth of HCT116 cells. These results indicate that Rottlerin and Lovastatin both possess anti-proliferative abilities *in vitro* and retard the growth of HCT116 cells.

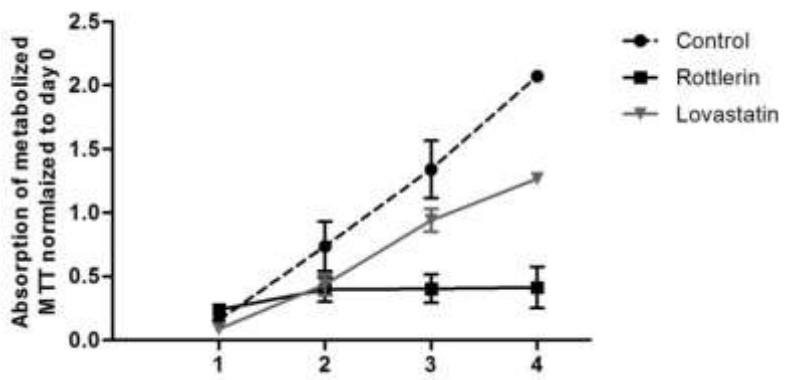


Fig. 4.19 Effect of Rottlerin and Lovastatin on anchorage-dependent cell proliferation. HCT116 cells were treated with daily doses of 2.5 µM Rottlerin or with 5 µM Lovastatin and the proliferation was analyzed daily with MTT assay for 4 days consecutively. Control cells were treated with equivalent amount of DMSO. The values were normalized to day zero values. Data represent mean ± SEM (n>3).

4.3.4.2 Rottlerin and Lovastatin arrest colony formation

Anchorage-independent growth of cells in soft agar is an important attribute of cancer cells representing cellular transformation, uncontrolled growth and metastatic behavior [161]. Anchorage-independent growth was investigated with the soft agar colony formation assay. Solvent-treated HCT116 cells were able to form large, clearly visible colonies within 12-14 days. In contrast, the size and number of the colonies formed were significantly reduced upon Rottlerin or Lovastatin treatment. Rottlerin treatment reduced the number of colonies to 56% of solvent control cells whereas treatment with Lovastatin reduced the number of colonies to 32% of solvent control cells (Fig. 4.20). Thus, to conclude, Rottlerin and Lovastatin possess both anchorage-dependent and anchorage-independent anti-proliferative abilities on HCT116 cells which might be partially explained by their specific inhibitory effect on MACC1 expression.

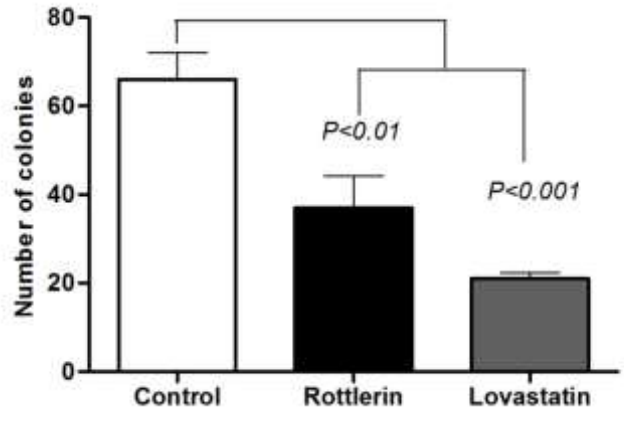
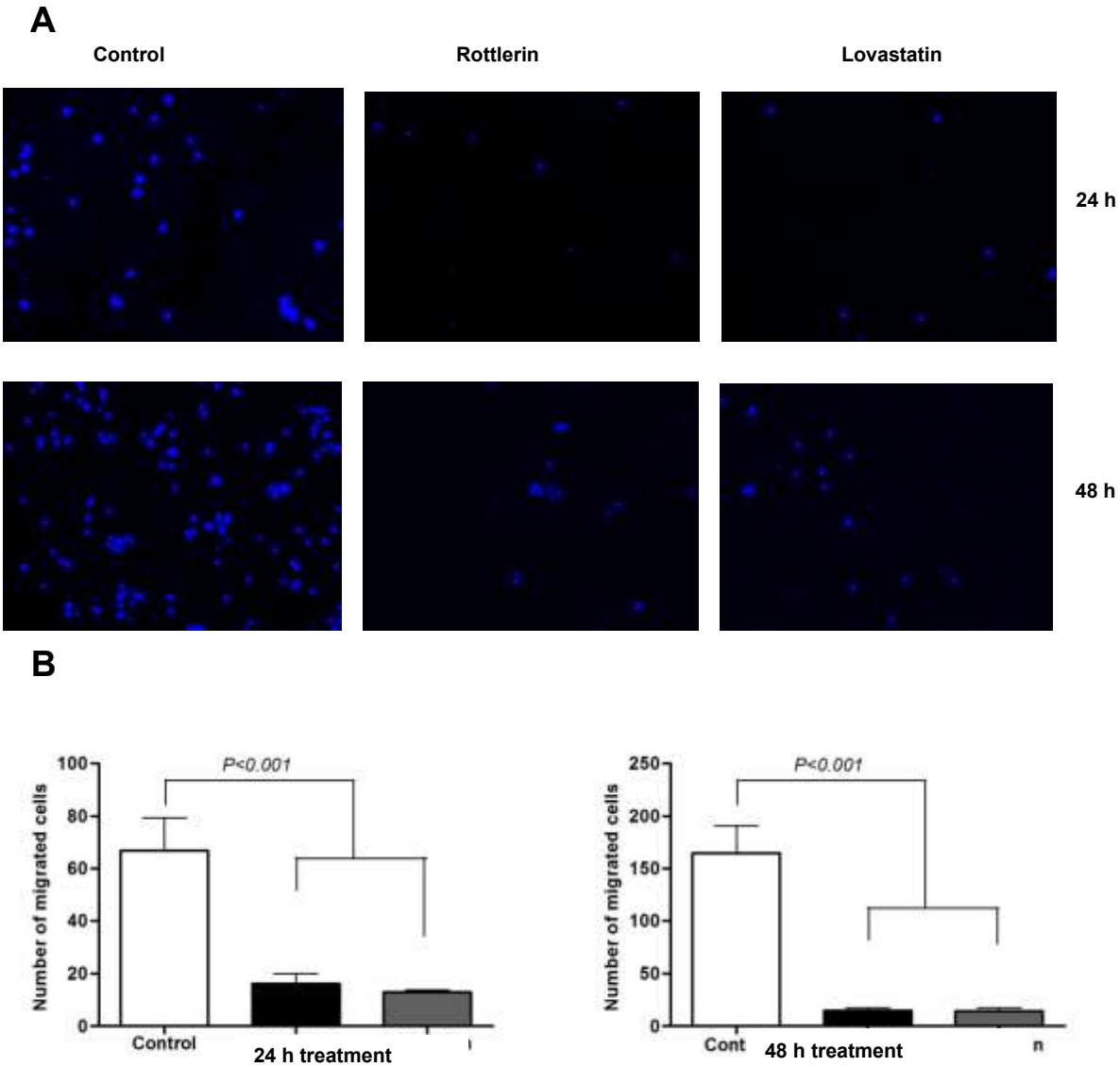


Fig. 4.20 Impact of Rottlerin and Lovastatin on anchorage indepen d-ent cell proliferation. Anchorage-independent growth of HCT116 cells upon treatment with 2.5 µM Rottlerin or 5 µM Lovastatin was analyzed by colony formation assay. Control cells were treated with equivalent amount of DMSO. Both small molecule inhibitors reduced the size and number of HCT116 cell colonies. Colonies were counted under light microscope on day 12-14. Data represent mean ± SEM (n>3).

4.3.4.3 Rottlerin and Lovastatin reduce cell migration

A major phenotype imparted by MACC1 is increased migration of the CRC cells [108, 113]. Thus, we investigated the effects of our small molecule MACC1 inhibitors on cell motility.

Migration was calculated at two different time points of 24 h and 48 h. The number of migrated cells over a time point of 48 h was more than two-fold as compared with 24 h time point. Further, the number of migrated cells upon treatment with 2.5 μM Rottlerin or 5 μM Lovastatin for 24 h was reduced up to 24% and 20% of solvent-treated cells, respectively, and was further reduced to 10% and 8.6%, when treated for 48 h (Fig. 4.21 A, B). As shown in figure 4.19, Rottlerin and Lovastatin at 2.5 and 5 μM respectively have no effect on proliferation at 24 h, suggesting that the effect on migration at this time point is mostly independent of the proliferation. On the other hand, proliferation starts to decrease significantly at 48 h post treatment with Rottlerin and
Lovastatin, suggesting that the effect we observe on migration at 48 h is probably a synergistic effect of migration as well as proliferation.



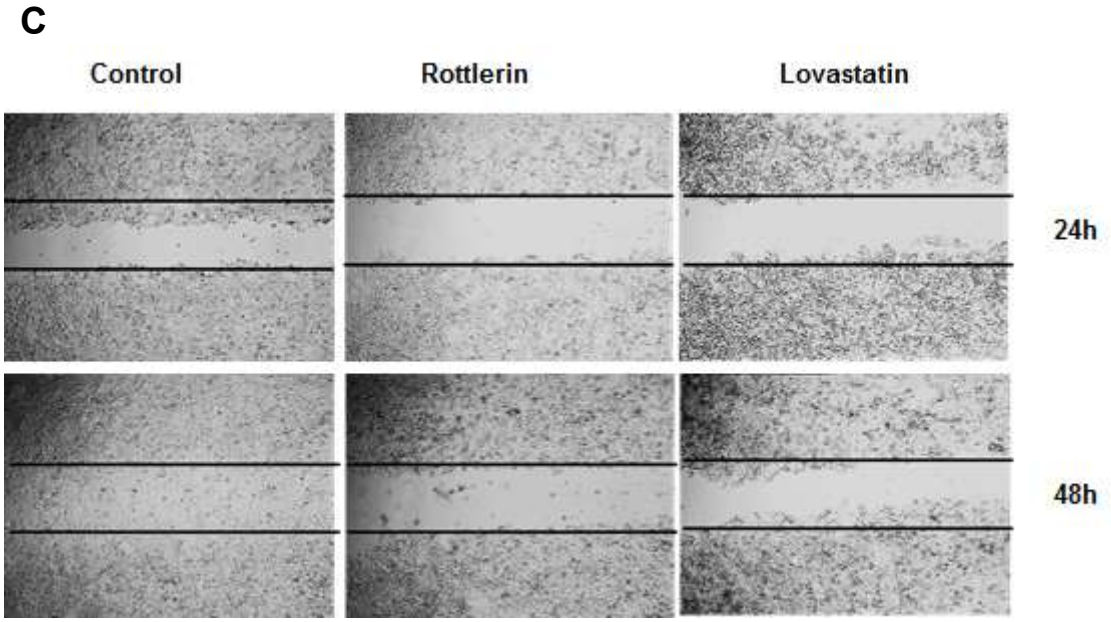


Fig. 4.21 Effect of Rottlerin and Lovastatin on cell migration. A) HCT116 cells were treated with 2.5 µM Rottlerin or 5 µM Lovastatin for 24 h and 48 h and cell migration was measured with the Boyden chamber assay. Migrated cells were fixed with 100% methanol and stained with DAPI. Control cells were treated with equivalent amount of DMSO. The representative images are shown. B) The migrated cells from five fields were counted manually under a fluorescent microscope and represented in the bar graph. Data represent means ± SEM (n=2). C) Directed migration of Rottlerin, Lovastatin or solvent-treated HCT116 cells was analyzed by wound healing assay. Microphotographs were taken 24 h and 48 h post treatment with 10x magnification. Assay was performed three times; one representative picture is presented here.

Similar effects were observed for directed-migration of HCT116 cells analyzed by wound healing assay as previously described [162]. When cells were treated only with solvent, HCT116 cells were able to infiltrate the wound and close it completely within 48 h. Treatment of HCT116 cells with 2.5 µM Rottlerin or 5 µM Lovastatin inhibited the wound closure as compared to the solvent treated cells (Fig. 4.21 C).

In summary, it can be concluded that both small molecule inhibitors are able to decrease MACC1 expression and, thereby, arrest primary attributes associated with this gene, i.e. migration and proliferation, making them suitable candidates as metastasis inhibitors.

4.3.5 Small molecule inhibitors interfere with the MACC1 transcriptional regulation

4.3.5.1 Rottlerin and Lovastatin inhibit Sp1 expression

We have demonstrated in our result section 4.1 that AP-1 and Sp1 play an important role in governing MACC1 transcriptional regulation. Thus, after validating MACC1 inhibitors *in vitro* we were interested in studying the mechanism by which our small molecule inhibitors restrict MACC1 transcriptional regulation. With the already acquired knowledge of the roles that

AP-1 and Sp1 play in transcription of the MACC1 gene, we would like to assess the effect of our inhibitors on these transcription factors regulating MACC1. Therefore, we first started by analyzing their effect on Sp1 expression. Treatment of HCT116 cells with Rottlerin and Lovastatin significantly inhibited the Sp1 mRNA levels by 57% and 23%, respectively, as compared with the solvent treated cells. These effects were more prominent as reflected in the Sp1 protein levels upon drug treatment as demonstrated by Western blotting. Thus, the inhibition of MACC1 by these inhibitors could be partially explained via decrease in Sp1 levels, thereby reducing transcription of the MACC1 gene.

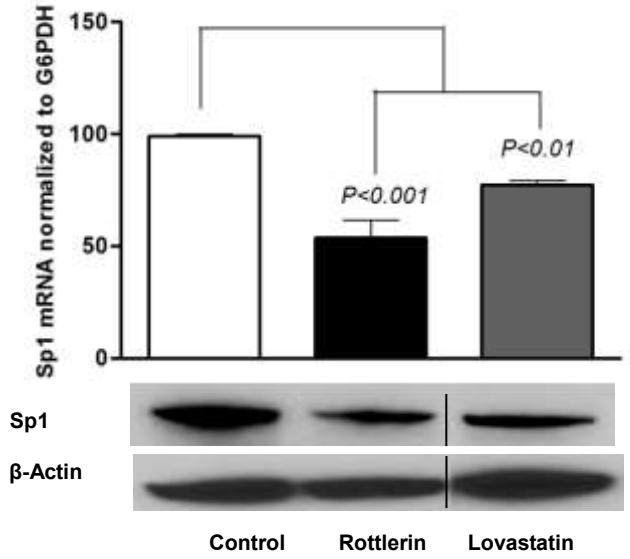


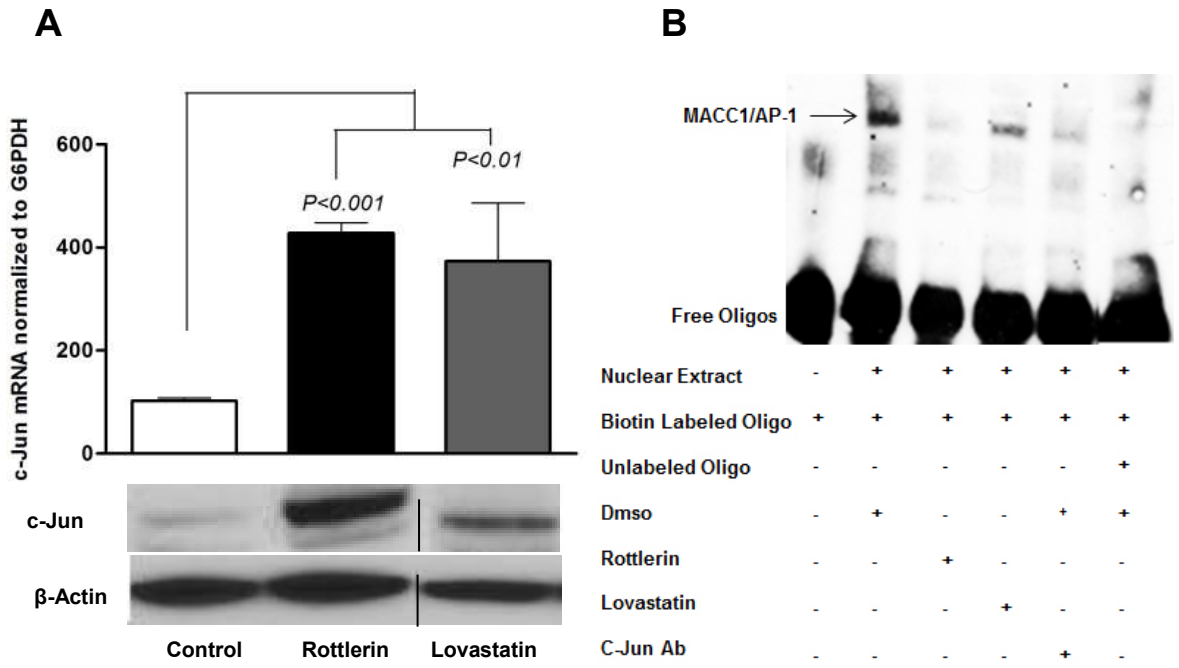
Figure 4.22 Effect of Rottlerin and Lovastatin on Sp1 expression. HCT116 cells were treated with 2.5 µM Rottlerin or 5 µM Lovastatin for 24 h and Sp1 mRNA and protein level were analyzed by qRT-PCR and Western blot, respectively. Control cells were treated with equivalent amount of DMSO. Black Line represents grouping of data after removal of an extra control lane. Data represent mean ± SEM (n=3).

4.3.5.2 Small molecule inhibitors hinder the binding of c-Jun with the MACC1 promoter

Surprisingly, treatment of HCT116 cells with Rottlerin and Lovastatin showed increased levels of c-Jun expression both at mRNA and protein levels (Fig. 4.23 A). Therefore, we hypothesized that these small molecule inhibitors may act within the nucleus and obstruct the binding of AP-1 with the MACC1 promoter. This hypothesis was tested by EMSA, using biotinylated oligonucleotides encompassing AP-1 binding site of the MACC1 promoter as described earlier. In solvent-treated HCT116 cells, signal shifts were observed which were caused by binding of c-Jun to the oligonucleotides as consistent with our previous findings (Fig. 4.4). Exposure of HCT116 cells to Rottlerin and Lovastatin interrupted the binding of AP-1 with the MACC1 promoter (Fig. 4.23 B). The specificity of the AP-1/MACC1 promoter

complex was verified by the addition of c-Jun antibody leading to disappearance of the shift (Fig. 4.23 C).

Similar results were found with ChIP assay (Fig. 4.23 D). Solvent-treated cell extracts showed two fold more enrichment of MACC1 promoter sequence after c-Jun immunoprecipitation as compared with Rottlerin or Lovastatin treated cell extracts. These results indicate that small molecule inhibitors inhibit AP-1/MACC1 promoter binding and thereby restrict MACC1 gene transcription.



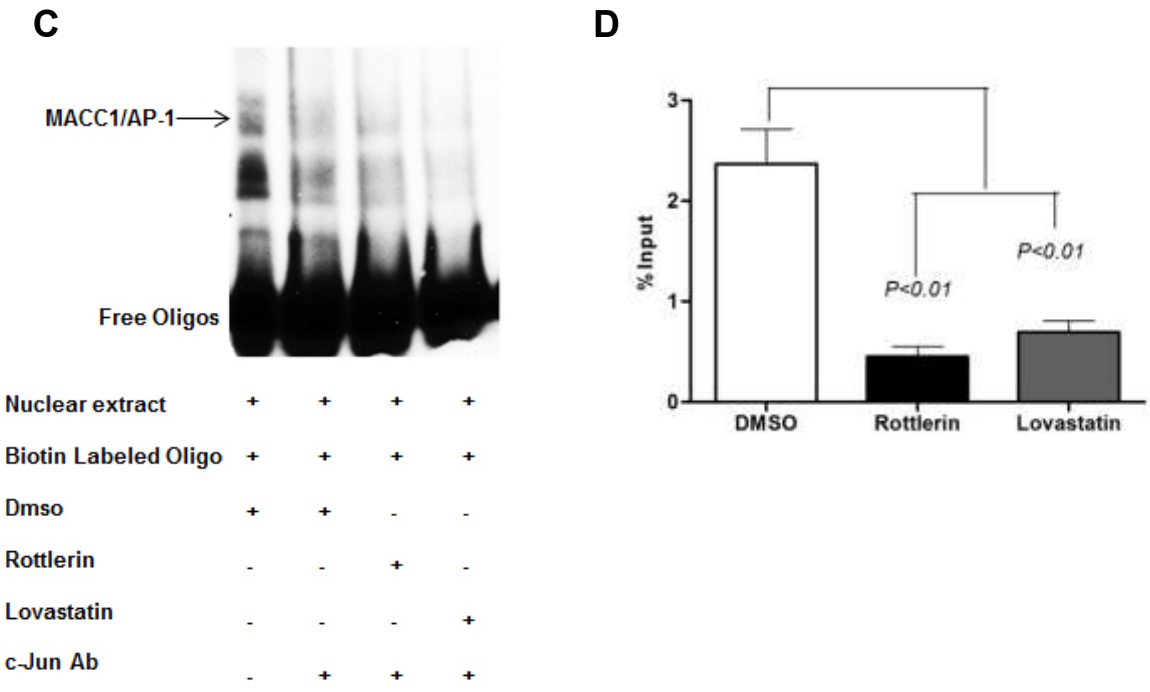


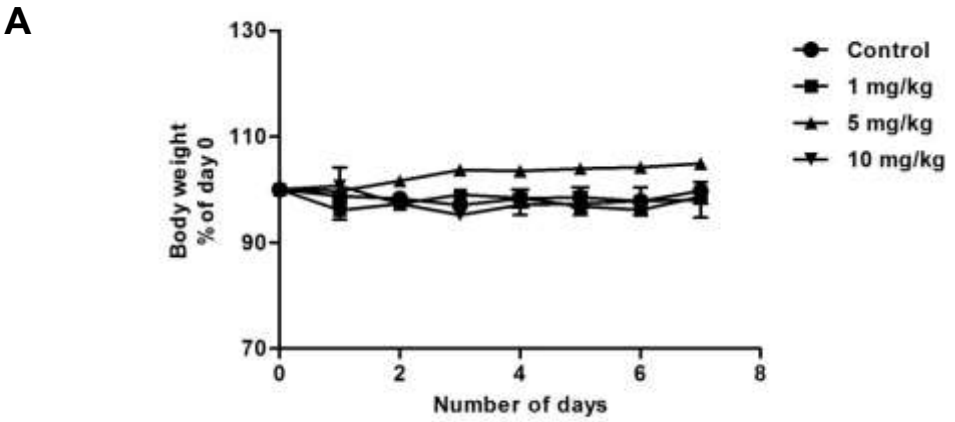
Figure 4.23 Effect of Rottlerin and Lovastatin on the binding of c-Jun to the MACC1 promoter. A) HCT116 cells were treated with 2.5 µM Rottlerin or 5 µM Lovastatin for 24 h and c-Jun mRNA and protein level were analyzed by qRT-PCR and Western blot, respectively. Black line represents grouping of data after removal of an extra control lane. B) EMSA was performed with equal amounts of nuclear extracts isolated from the HCT116 solvent-control and treated cells followed by incubation with 5' biotin labeled MACC1 promoter oligonucleotides flanking binding sites specific for AP-1. A reaction with 100x molar excess of unlabeled competitor sequence was also carried out indicating the specificity of the protein-DNA complexes. The reactions were analyzed by polyacrylamide gel electrophoresis. C) For supershift analysis, the nuclear extracts were incubated with biotin labeled oligonucleotides along with antibodies for c-Jun. Representative figure from two independent experiments is depicted. D) Equal amounts of HCT116 solvent-control and treated cells chromatin was immunoprecipitated with antibodies for c-Jun and was quantified by qRT-PCR, using primers for the MACC1 promoter. The results were plotted as percentage of input. Data represent mean ± SEM (n=3)

4.3.6 Rottlerin inhibits tumor growth in CRC xenograft mice

In vitro studies demonstrated a strong potential of Rottlerin as an inhibitor of MACC1 and justified the need to access its anti-proliferative and anti-migratory abilities *in vivo*. Thus, we analyzed the anti-cancerous and anti-metastatic effects of this small molecule inhibitor using xenografted mice. Intrasplenic transplantation of HCT116 cells into SCID mice was used as a model to analyze the effect of Rottlerin on primary tumor and liver metastases [108].

4.3.6.1 Rottlerin application *in vivo* caused no toxic side effects

For dose-finding experiments in human CRC xenografted mice, NOD-SCID mice were intrasplenically injected with HCT116/LUC cells and were intraperitoneally treated with daily doses of 1 mg/kg, 5 mg/kg or 10 mg/kg of Rottlerin or the respective amount of solvent, in cooperation with PD Dr. Iduna Fichtner (MDC). Body weight was measured as a first indicator for toxic side effects [163]. Body weight of the mice treated with the highest concentration of 10 mg/kg Rottlerin did not differ from the solvent-treated control (Fig. 4.24 A). Thus concentration of 8 mg/kg was selected for further experiments.



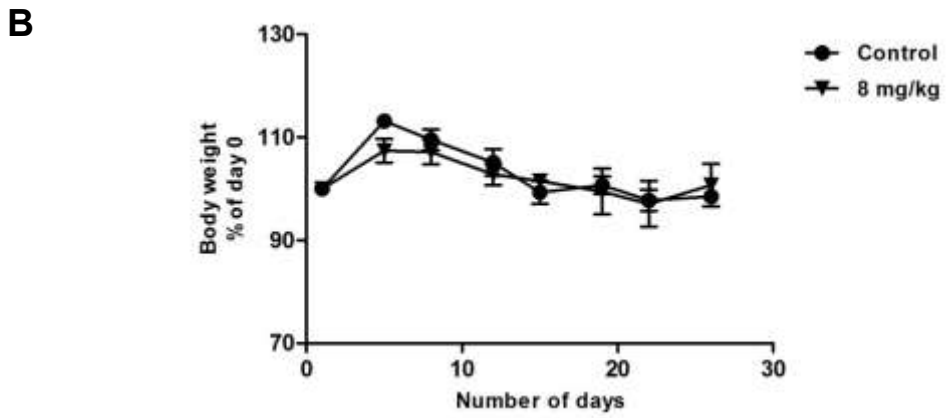


Fig. 4.24 Evaluation of Minimal tolerable dose (MTD) of Rottlerin for *in vivo* administration in mice. Indicated concentrations of Rottlerin were injected daily intraperitoneally into NOD-SCID mice bearing an intrasplenal HCT116/LUC tumor for seven days. Body weight was determined as an indicator for toxic side effects. Data represent mean ± SD (2 animals/group) (B) No difference in weight loss was observed between solvent or Rottlerin (8 mg/Kg)-treated mice, over a period of 27 days. Data represent means ± SD (4-5 animals/group)

4.3.6.2 Rottlerin restricted MACC1 expression and tumor growth *in vivo*

Bioluminescence imaging presents a non-invasive tool to assess tumor growth and development over time and is a state of the art technique in cancer research [164]. To track the growth of the human CRC cells in the immune-deficient mice, HCT116/LUC cells were used. These cells express MACC1 endogenously along with a CMV-promoter controlled firefly luciferase gene. This enzyme allows the oxidation of D-Luciferin, which leads to the emission of light. To monitor the effect of Rottlerin on tumor growth *in vivo*, HCT116/LUC cells were intrasplenically injected into NOD-SCID mice and the animals were treated daily with 8 mg/kg Rottlerin or the respective amount of solvent. A lateral signal became visible on day 5 post-transplantation in solvent- and Rottlerin-treated mice, which was assigned to the spleen (Fig. 4.25 A). This spleen tumor signal increased over time in the solvent-treated group whereas in the case of Rottlerin-treated mice, the growth was much slower starting from the beginning and the difference became clearly visible on day 19. Furthermore, on day 27, when the signal crossed the threshold value in the intensity bar in solvent-treated group, the Rottlerin treated mice still had signals within the range. However, solvent-treated control mice upon ventral imaging revealed almost no signal in the liver region on day 27 indicating that liver metastases had not been formed in these tumors. The experiment was terminated on this day and the animals were sacrificed. On isolating the organs, we clearly observed that in the Rottlerin treated group, the spleen tumor was much smaller as compared to solvent treated (Fig. 4.25 B). Moreover, the reduction in spleen weight (Fig. 4.25 C) in the Rottlerin-treated group as compared with solvent control group further strengthens our

finding. However, livers from the control group were mostly clean with no or only tiny tumor outgrowths (Fig. 4.25 B).

In order to ascertain that the drug is inhibiting MACC1 levels *in vivo*, we isolated RNA from spleen and performed qRT-PCR. Our PCR results indicate that Rottlerin is strongly inhibiting MACC1 expression (Fig. 4.25 D) thereby restricting the primary tumor growth.

Thus, to summarize, Rottlerin at the dose of 8 mg/kg inhibited MACC1 levels and restricted the tumor growth *in vivo*. However, its effect on liver metastasis cannot be speculated from our studies as no liver signals were observed in control mice until day 27.

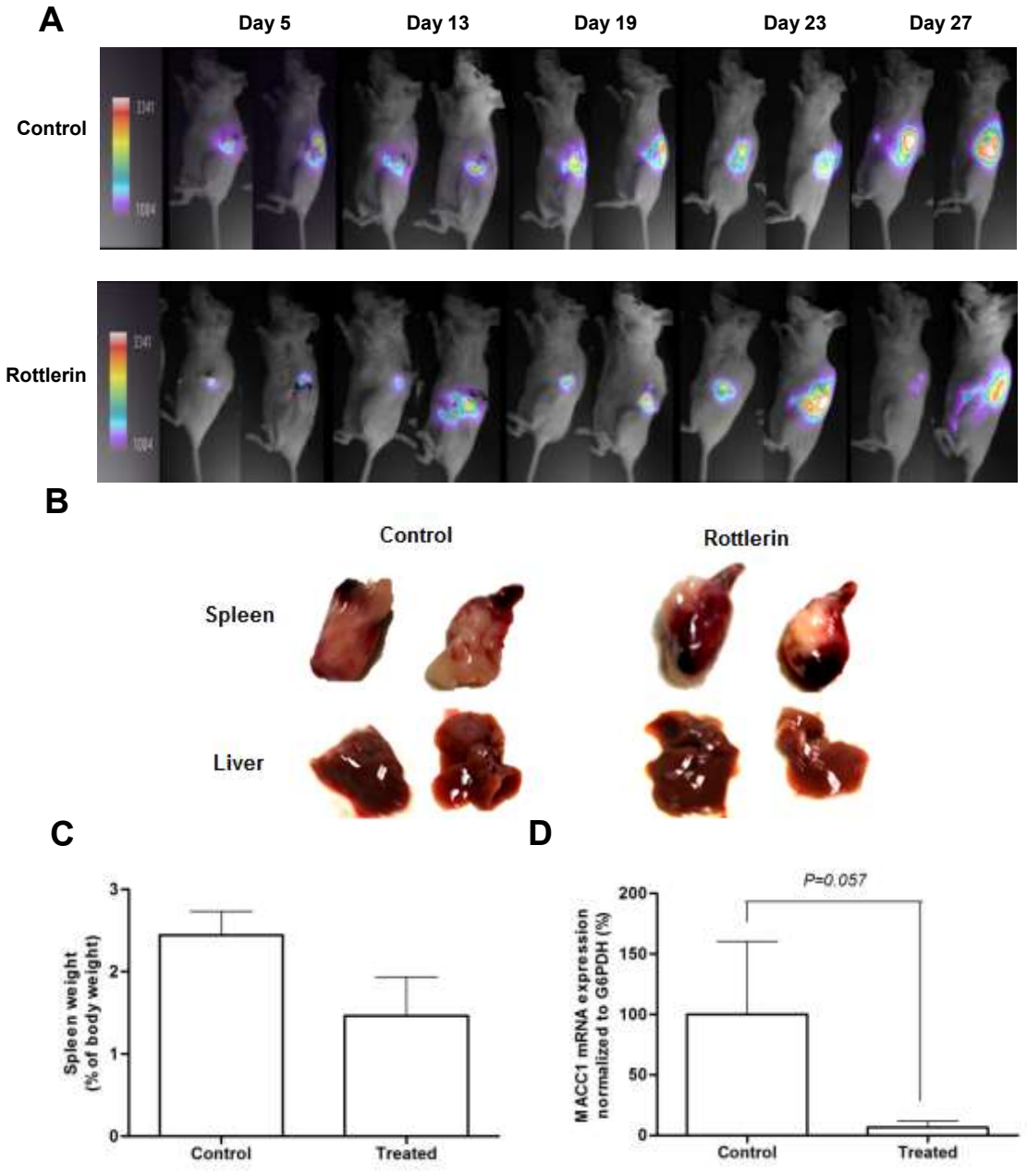


Fig. 4.25 Effect of Rottlerin in CRC xenografted mice as monitored by non-invasive bioluminescence imaging. NOD-SCID mice were intrasplenically transplanted with HCT116/LUC cells and mice were treated intraperitoneally with 8 mg/kg Rottlerin every day. Bioluminescence was measured by intraperitoneal application of 150 mg/kg D-Luciferin and sequence exposure of 1 min for 10 times in the NightOWL LB 981 system. A) The lateral signal from the spleen tumor as monitored via imaging over time in solvent- and Rottlerin-treated mice. B) *Ex vivo* imaging of isolated organs confirmed the signals seen above. C) Total body weight and spleen weight was measured on day 27 after xenografting and spleen weight in percentage of body weight was calculated. D) On day 27, the animals were sacrificed and the spleens were shock frozen. RNA was isolated from this frozen spleen and MACC1 mRNA levels were determined using qRT-PCR. Data are representative of four animals per group.

5. DISCUSSION

Over the last few years, a substantial increase in five-year survival rate of the CRC patients has been observed, but this increase was primarily restricted for patients with localized cancer and no progress was observed for patients with advanced disease [1]. Understanding of the heterogeneous nature of cancer has brought the emergence of a new era of personalized medicine, embracing a more stratified approach to cancer management. The success of personalized therapy demands a clear knowledge of the molecular underpinnings of the disease defining the clinical characteristics of different patient sub-populations with different outcomes in relation to a given treatment.

Almost five years ago, MACC1 was identified as a prognostic biomarker and as an indicator of pathogenic process leading to CRC metastasis [2]. Since then, various follow-up studies had emphasized the importance of MACC1 in progression of CRC and had also provided hints towards its role as predictive marker [3-10]. However, the characteristics of the MACC1 promoter, as well as the mechanisms governing MACC1 gene regulation remained largely unknown. Of note, targeting MACC1 with shRNA in xenografted mice demonstrated a reduction in the rate of tumor progression and metastasis formation suggesting that inhibition of MACC1 provides a promising therapeutic strategy for restricting CRC progression and metastasis intervention [132]. Though, the pharmacological targeting of MACC1 with small molecule chemical compounds and their impact on CRC progression has not been addressed so far.

Therefore, this study has addressed MACC1 gene regulation through identification of the MACC1 promoter followed by studying the transcription factors governing transcription of the MACC1 gene. This knowledge was then translated to identify and investigate two novel MACC1 transcriptional inhibitors in CRC cells leading to reduced MACC1 expression and thus, consequently reduced proliferation and migration *in vitro* and tumor progression *in vivo*. Our study broadens the existing knowledge underlying the molecular mechanism behind MACC1-induced-tumor progression and metastasis formation and further strengthens its role as a therapeutic target.

5.1 Transcriptional regulation of the MACC1 gene

5.1.1 Identification and relevance of the MACC1 core promoter region

Analysis of the 5' flanking region upstream of the TSS in the MACC1 gene, revealed that the genomic fragment spanning nucleotides -992 to -18 bp contains the promoter region for the MACC1 gene, which can be further extended up to -1992 bp as both MACC1 long range promoter constructs (MACC1p-992 and MACC1p-1992) were able to drive transcription. However, luciferase assays performed with 5' serially truncated MACC1p-992 constructs revealed a minimal essential core promoter region spanning between the nucleotides from -426 to -18 bp. This core promoter region possessed a comparable promoter activity as the long range promoter itself indicating that it contains all information required to drive transcription of MACC1. Of note, we found high degree of similarity in this core promoter region between human and mouse MACC1 promoter sequence. This core promoter region might not be only responsible for initiation of transcription but it might play a role in tethering together all the elements that are required for the basal activation of the MACC1 gene. These are often called as regulatory promoters. However, the long range promoter might be responsible for tissue specific expression or might play a role in protecting the gene from the positive and negative influences exerted by the chromatin at the site of integration very often termed as positional effect [165]. In addition, distant genomic elements namely enhancers, repressors or insulators can be located upstream, within introns, or downstream of the regulatory promoter [166]. Furthermore, multiple alternative promoters can dictate the strategy of gene expression as it is the case of the well characterized human Bcl2 gene which possess a Sp1 binding site and no TATA box and demonstrate multiple sites of transcription initiation. Nevertheless, the aim of our study was to investigate the regulatory promoter of the MACC1 gene. Distant genomic elements and presence of alternative promoters for the MACC1 gene needs to be investigated in the future.

5.1.2 Exploration of the transcriptional regulatory machinery of the MACC1 gene

In silico and *in vitro* studies of the core promoter region revealed the presence of potential binding sites for various transcription factors including AP-1, Sp1 and C/EBPs. AP-1 expression and activity is shown to be altered in many cancers, including CRC [167]. The fact that AP-1 has increased activity in cancer cells and is often associated with their transformed phenotype suggests that it may be fundamental to the process of oncogenesis [168-170]. We propose that MACC1 regulation via AP-1 imparts another dimension to AP-1 as a target for cancer therapy.

There is also emerging evidence that Sp1 protein expression may be a critical factor in tumor development, growth and metastasis [156]. Sp1 protein is highly expressed in the nuclei of gastric, pancreatic, breast, and thyroid tumors compared to their normal tissues [152, 171, 172]. Moreover a study showed that the survival of patients with high Sp1 expression was significantly decreased compared to patients with weak to non-detectable Sp1 expression [156]. There have also been studies showing that AP-1 and Sp1 together regulate transcriptional activation of various human genes encoding leukocyte integrin CD11c [173], Involucrin [174], Loricrin [175], and the metastasis gene VIL2 (Ezrin) [176]. Furthermore, C/EBPs are also overexpressed in colorectal tumors [177-179]. Their role in regulating gene expression via regulating AP-1 has also been demonstrated in several different cell models [180-182]. Specific involvement of AP-1, Sp1 and C/EBP in transcription of the genes involved in proliferation and metastasis aroused our interest to study their role in regulating MACC1. In order to validate the functional relevance of the binding sites for these transcription factors on the MACC1 promoter, we performed site directed mutagenesis studies. The results demonstrated that AP-1, Sp1 and C/EBP binding sites contribute to the transcriptional activity of the MACC1 promoter. EMSA and ChIP experiments further confirmed the physical association of these elements with the MACC1 promoter.
Taken together, these three transcription factors bind to the MACC1 promoter and might cooperatively regulate MACC1 expression.

5.1.3 Regulation of MACC1 expression and MACC1- associated motility by AP-1 and Sp1

RNAi strategy using target-specific predesigned siRNA for knocking down the transcription factors regulating MACC1 assured their effect on MACC1 expression levels. Chemical inhibitors for these transcription factors could be used also but the limitation for specifically targeting these transcription factors would have still prevailed. It remains possible that these transcription factors affect the expression of many additional genes or transcription factors already implicated in migration and metastasis. Consequently, the decreases in MACC1 expression that follow the AP-1, Sp1 or C/EBP silencing might be direct or indirect through regulation of other transcription factors. For C/EBPs, it is known that either post-translational modification or alternative translation initiation result in diverse isoforms with opposing physiological roles thereby forming a C/EBP interactome which determines cell fate. Consequently, the role of C/EBPs in the regulation of MACC1 and MACC1-induced functional phenotype might depend on these epigenetic changes regulating C/EBP itself. Thus, we then focused on AP-1 and Sp1 and demonstrated that siRNA for these

transcription factors induced low migratory abilities in CRC cells. The rescue experiment carried out by overexpressing MACC1 ectopically further convinced us that the low migratory abilities observed upon knocking down c-Jun and Sp1 are MACC1-specific. Therefore, AP-1 and Sp1 regulate MACC1 expression and MACC1-associated cell motility.

5.1.4 Expression analysis of c-Jun and Sp1 in patients with variable levels of MACC1

The association of AP-1 and Sp1 with MACC1 was then confirmed in clinical specimens. We found a strong positive correlation of MACC1 expression with c-Jun and Sp1 expression in colorectal tumors. Our findings further reflected the interdependence of the AP-1 and Sp1 with MACC1 levels linked to development of distant metastases. Patients with high MACC1 expression in their primary tumors showed high c-Jun as well as high Sp1 levels. Remarkably, high levels of AP-1 as well as of Sp1 were also found in those tumors that later on developed distant metachronous-metastases. In the future, the study on a larger sample size might further emphasize the correlation of MACC1 with the two transcription factors and might also be able to clarify whether MACC1 along with c-Jun or Sp-1 increases the prognostic power as compared to MACC1 alone.

5.1.5 Relevance of GIPC1 in regulating MACC1

GIPC1 family constitutes the PDZ domain bearing members, GIPC1, GIPC2 and GIPC3. GIPC1 is a scaffold protein involved in trafficking of various trans-membrane proteins, signaling and recycling of receptor tyrosine kinases (RTKs), G protein-coupled receptors (GPCRs), integrin and thus regulate various cellular processes such as proliferation, planar cell polarity, cytokinesis and migration. One of the major pathologies associated with the GIPC1 is cancer. GIPC1 was found to be overexpressed in breast, ovarian, colorectal, gastric and pancreatic cancers [183-187]. A study reported that GIPC1 is required for pancreatic tumor growth in nude and SCID mice via reduction in the expression of insulin-like growth factor receptor I (IGF-1R) [188]. Moreover, GIPC1 knock down gene signature was correlated with a number of breast and ovarian cancer phenotypes and clinical outcomes, including patient survival as investigated using publically available breast and ovarian cancer microarray datasets [183]. Considering the promising role of GIPC1 in oncogenic transformation, we hypothesized the connection between MACC1 and GIPC1. Stable knockdown of GIPC1 in a high MACC1 expressing CRC cell line resulted in a decrease in the MACC1 expression. Interestingly, the decrease in MACC1 levels was due to a decrease in the MACC1 promoter activity in GIPC1 knockdown cells compared with control cells. This indicated that GIPC1 governs a part of the MACC1 promoter activity. Thus, we carried out

ChIP and EMSA experiments to depict the physical interaction of GIPC1 with the MACC1 promoter, and the results suggested that, indeed, GIPC1 binds to the MACC1 promoter and regulates its activity. This was the first study elucidating the role of GIPC1 as a transcription factor. Additionally, we demonstrated that GIPC1 might bind to the first 133 bp upstream of the TSS, but it remains unclear whether GIPC1 act as an accessory molecule to mediate transcription of the MACC1 gene or directly binds to the MACC1 promoter sequences and plays an essential role in oncogenic transformation mediated by MACC1.

5.1.6 Summary and scope: MACC1 promoter study

Our present work led to the identification of the MACC1 regulatory promoter and transcription factors which bind to it and contribute to MACC1-associated metastasis in CRC (Fig. 5.1). Until now, the majority of the studies on the biomarker MACC1 were associated with the clinical implication of this gene. Our results contribute to the understanding of the molecular interactions of AP-1, Sp1, C/EBPs and GIPC1 with the gene promoter. Oncogenic transcription factors such as AP-1 and Sp1 are particularly promising therapeutic targets since they often have increased expression and activity in a variety of cancers. Associated with this is the fact that they mediate signals coming from multiple different pathways, hence inhibiting their function will likely to interfere with the function of numerous signaling molecules which could have favorable or unfavorable outcome on the cancer patient. A relevant approach could be the identification of a drug which rather than completely inhibiting c-Jun or Sp1 expression, impairs its binding with the specific gene promoter, in our case, MACC1, and thus arrest oncogenic transformation. It has been shown that different C/EBP isoforms might have different roles in tumor formation and progression, C/EBP-α seems to be a general tumor suppressor in both hematopoietic and non-hematopoietic cell types whereas C/EBP-β might both inhibit and promote cell cycle progression, depending on the cellular context and C/EBP-β forms present [177]. Therefore, the role of C/EBPs in the context of MACC1 regulation warrants further elucidation. Lastly, therapeutic implication of GIPC1 in cancer has already been speculated [183] and our study strengthens its promise as a new target for cancer.

Thus, the knowledge acquired from our present study might provide a rationale for the development of various intervention strategies targeting MACC1 expression, thereby translating this new knowledge into clinical applications.

Fig. 5.1 Regulation of the MACC1 promoter. Schematic representation of the MACC1 promoter and its regulation by GIPC1, Sp1, C/EBP and AP-1 transcription factors.

5.2 MACC1 as a new target for advanced colorectal cancer therapy

Cancer biomarkers bear multi-faceted aspects in cancer intervention. They can be used for cancer diagnosis, risk and prognosis assessments and for predicting effectiveness of the particular therapy. In addition, some biomarkers can be used as targets for cancer therapy. As stated by Zhiyuan Shen, biomarkers can be of two types, one are simply “messengers”, and that do not directly contribute to cancer growth and hence cannot be targeted for therapies. While the second class of biomarkers are called as “driver” or “conspirator” biomarkers that directly contribute to tumor growth and hence can be exploited for their efficacy as a therapeutic target [189]. As mentioned above, MACC1 has been established by various studies as a prognostic biomarker for colorectal cancer metastasis [108-111,114,115,117]. It has also been demonstrated in these studies along with many other [117, 124, 125, 127, 129, 132, 190, 191] that MACC1 levels can act as a decisive driver for the switch between adenoma to carcinoma and thus initiate cancer progression and ultimately metastasis. Interestingly, MACC1 was also found to be up-regulated in intestinal stem cell populations with Lgr5-GFP high expression indicating that MACC1 in the stem cell population could be one of the potential reasons for tumor relapse [134]. Therefore, in our study, we hypothesized the potential of MACC1 for targeted therapy and thus aimed to target MACC1 to intervene in CRC progression.

5.2.1 HTS implicated in identification of novel small molecule MACC1 inhibitors

As discussed earlier, patients with elevated levels of MACC1 in their primary tumors showed higher risk of metastasis, cancer recurrence and low metastasis free survival. In addition, xenografted mice treated with shRNA MACC1 showed reduction in primary tumor and liver metastasis [132]. RNA interference as therapeutics modality is still under clinical trial and has not been adopted because of its off target effects, ability to trigger type I interferon responses, competition with cellular RNAi components and most importantly, for the challenges it impose on the effective delivery *in vivo*. As an alternative, we aimed at the investigation of chemical compounds that can inhibit MACC1 and MACC1-induced

oncogenic transformation and thereby, reduce the risk of patients to progress towards advanced stages of CRC.

Inhibiting the expression of a gene could be achieved via several mechanisms. We could design inhibitors which bind reversibly or irreversibly to the protein of interest and blocks its function. Others might be epigenetic inhibitors, transcriptional or post translational inhibitors. Structural insight and the protein structure of the MACC1 gene are still unknown. Thus, we designed a strategy to investigate chemical compounds that can inhibit transcription of the MACC1 gene by manipulating its promoter activity, which was only possible because of our previous identification of the MACC1 promoter.

In order to identify chemical drugs targeting MACC1 transcription, we performed HTS with a Chembionet library of more than 30,000 compounds using HCT116 cells stably transfected with MACC1 promoter-luciferase constructs. Out of 30,000 compounds, 97 compounds, which included seven pharmacologically active and 90 novel compounds, emerged as potential candidates which specifically inhibited MACC1 promoter activity. We selected pharmacologically active compounds which inhibited MACC1 promoter activity because of their better chances in terms of clinical development. This led us to the identification of two MACC1 inhibitors namely Rottlerin and Mevastatin.

5.2.1.1 Rottlerin and known biological activities

Rottlerin is a plant derived compound obtained from the reddish brown powder covering the berries of the *Mallotus philippinensis* (also known as Kamla tree). This tree is usually found in the tropical regions of India, Philippines, Southeast Asia and Australia. It is a spectacular plant with different parts of it possessing different biological activities and is used as traditional medicines. According to Ayurveda, the leaves of this tree are a good appetizer. Its fruit bears heating, purgative, anthelmintic, vulnerary, detergent, carminative, alexiteric properties. Phloroglucinol derivatives obtained from the fruit of this plant possess anti-allergic properties [192] whereas seed extract showed antifertility actions [193]. The reddish brown powder (Rottlerin) is locally used for coloring textile and is used for centuries as an old remedy against tape worm infection because of its laxative effect, indicating its safety profile as a drug [194].

Rottlerin is also known as Mallotoxin and its molecular formula is $C_{30}H_{28}O_8$ with a molecular weight of 516.5 g/mol. IUPAC name for Rottlerin is (E)-1-[6-[(3-acetyl-2,4,6-trihydroxy-5-methylphenyl) methyl]-5,7-dihydroxy-2,2-dimethylchromen-8-yl]-3-phenylprop-2-en-1-one. It emerged as a compound of commercial importance following the study reporting it as a selective PKCδ inhibitor [195]. However there is an open debate on the selectivity of

Rottlerin. Rottlerin was demonstrated to uncouple mitochondrial respiration from oxidative phosphorylation, reducing cellular ATP levels and thereby, it non-specifically inhibits PKCδ kinase activity in cultured cells [196]. Nonetheless, many studies have continued to use and show consistent and convincing results using Rottlerin to inhibit PKCδ signaling [197, 198].

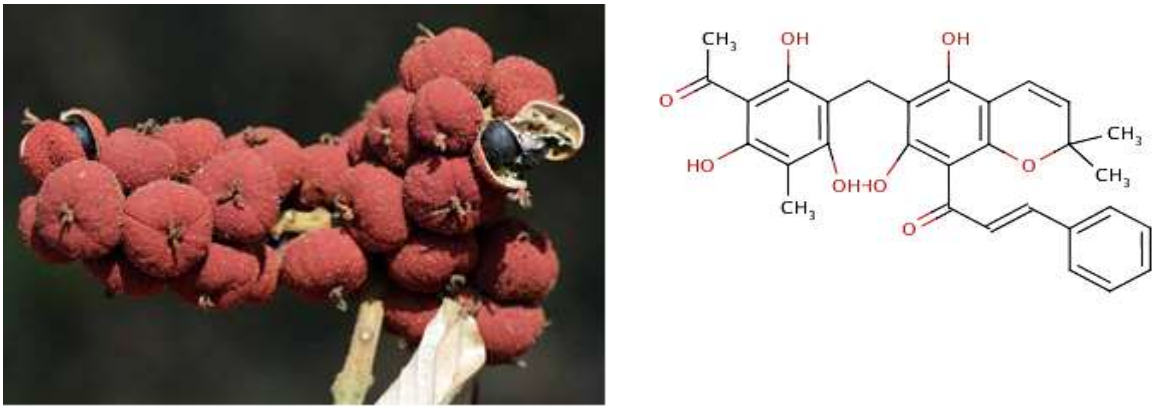


Figure 5.2: Fruit of Kamala tree and Rottlerin structure. (Adapted from biotik.org/India)

Apart from its anthelmintic and laxative effect against tapeworm, it has also shown potential as potassium channel (BKCa++) opener, thus reported to have cardio-protective effects after ischemic-reperfusion injury [199]. Rottlerin, through inhibition of PKCδ is reported to have a neuro-protective effect in an animal model of Parkinson's disease [200]. Additionally, it also possess anti-inflammatory properties [201] and very recently it was reported as a small molecule therapeutics for asthma [202].

Rottlerin exhibits diverse effects against various cancer cells. It can induce apoptosis or autophagy through different mechanisms in different cancer models [203-208]. Rottlerin is also shown to reduce cell motility and invasiveness via decreasing levels of integrin β1, FAK, paxillin, Rac-1, Rho GTPases [209-211] and MMP9 [212, 213]. Still, the mechanisms underlying the Rottlerin anti-cancer properties are relatively poorly understood and its role as an anti-cancer agent is not established. In this study, we explored a new target of Rottlerin and further established its potential as a therapeutic drug against CRC.

5.2.1.2 Statins and known biological activities

The second potential MACC1 transcriptional inhibitor that emerged from the HTS was Mevastatin. It belongs to the class of drugs “Statins” which reduce cholesterol levels by inhibiting the enzyme hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase. This inhibits the rate limiting step in the mevalonate pathway of cholesterol synthesis and is thus a useful drug to treat hypercholesterolemia [214]. Mevastatin (also known as Compactin) was the first

statin, isolated from *Penicillium citrinum*, which was found to have powerful inhibitory effect on HMG-CoA reductase [215-217]**.** Unfortunately, Mevastatin never came into the market due to its severe side effects and toxicity in dogs. However, in 1978 a new statin, Lovastatin, isolated from *Aspergilus terreus* was identified by Alfred Alberts and colleagues which was found to be effective in reducing blood cholesterol [218]. In 1987, it was approved for sale by the FDA as the first cholesterol-lowering drug. Subsequently, several other HMG-CoA reductase inhibitors like Simvastatin, Pravastatin, Fluvastatin, Atorvastatin, Cerivastatin and Rosuvastatin came into the market.

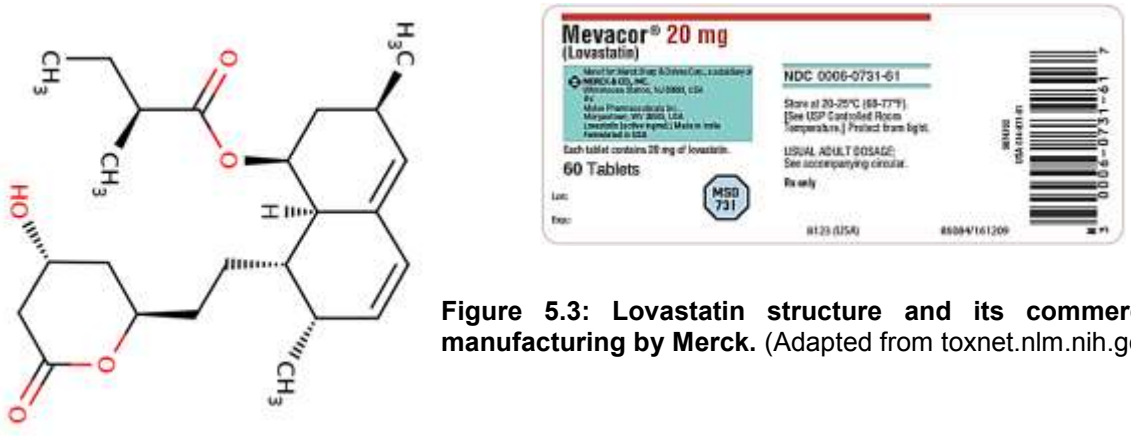


Figure 5.3: Lovastatin structure and its commercial manufacturing by Merck. (Adapted from toxnet.nlm.nih.gov)

Apart from the lipid-lowering potential of Statins, several studies have speculated its effect on tumor development through multiple mechanisms (Fig. 5.4). Remarkably, in the molecular Epidemiology of Colorectal Cancer study, including 1953 patients with CRC and 2015 controls, statin usage was associated with a 47% decrease in the relative risk of CRC (odds ratio, 0.50) [219]. However, the molecular mechanisms of the effects of statins against cancer are not completely elucidated. In our study, we investigated the role of Lovastatin in CRC progression mediated by MACC1 in order to strengthen its therapeutic value in oncology.

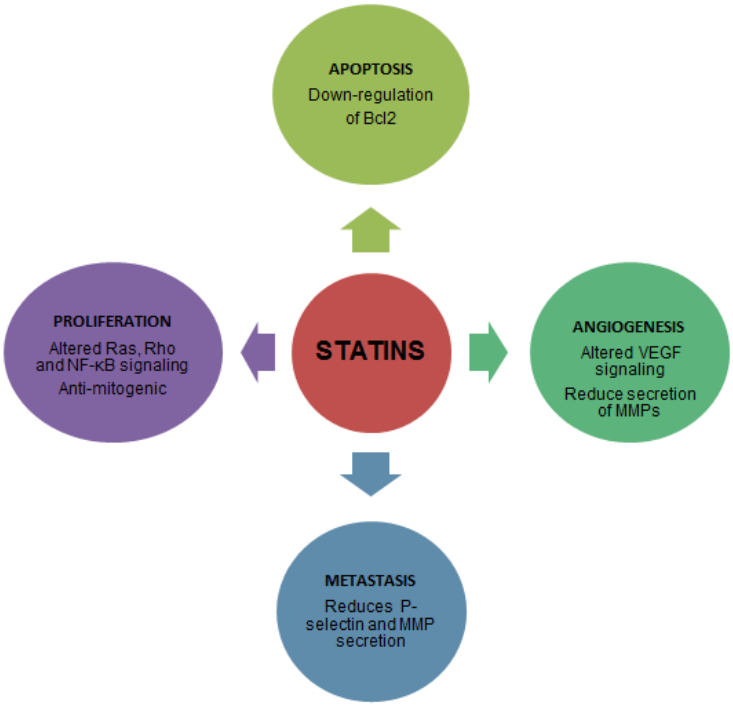


Figure 5.4: Potential mechanism of action of Lovastatin in Cancer. Modified after Kaushal et al. [220].

5.2.2 MACC1 inhibitors restrict MACC1 expression and MACC1 associated biological properties

Based on our HTS, we identified the first small molecule MACC1 inhibitors known so far, Rottlerin and Lovastatin. Both the compounds inhibited MACC1 promoter activity indicating that they exert their effect via impairing MACC1 transcription. Consequently, in this study it was shown that both small molecule inhibitors reduced MACC1 mRNA and protein expression in CRC cells but the effects were dependent on drug concentration, time of treatment and the cell line used. This is the first study demonstrating the effect of Rottlerin and Lovastatin on MACC1. In contrast to the remarkable drop in MACC1 mRNA levels observed upon treatment with Rottlerin after 18 h, the maximum inhibition of MACC1 mRNA by single dose of Lovastatin was observed at 36 h. However, at the protein level, we observed a time-dependent decrease in MACC1 starting from 24 h onwards upon treatment with either of the drugs, probably because of different *in vivo* half-lives of the MACC1 protein like many other proteins [221]. Consistent with our findings, both small molecule inhibitors did not affect exogenously overexpressed MACC1 levels controlled by a CMV promoter. Therefore, the effects on MACC1 expression are specific for endogenous MACC1 regulated by the MACC1 promoter.

Further, treatment of CRC cells with Rottlerin or Lovastatin inhibited anchorage-dependent and independent cell proliferation. Knock-down of MACC1 levels using RNA interference strategy was shown to restrict cell proliferation and tumorigenicity in nasopharyngeal, cervical, osteosarcoma, glioblastoma and gastric cancer cell lines [108, 128, 129, 222-224]. These studies have also shown that knock-down of MACC1 resulted in G0/G1 arrest and inhibition of Akt signaling. A decreased MACC1 expression upon treatment of CRC cells with Rottlerin and Lovastatin, thus, showed decrease of cell proliferation and colony formation. These effects could be an outcome of reduced MACC1 levels but the possibility of an independent mechanism contributing to decreased proliferation along with low MACC1 levels still prevails.

Additionally, MACC1 is a major regulator of cell motility [108, 109, 114, 117, 126-128, 130, 135, 190, 223]. Knock-down of MACC1 using RNAi experiments restricted cell migration and invasion. Though, a molecular mechanism elucidating the role of MACC1 in metastasis remains unclear. CRC cells upon treatment with Rottlerin or Lovastatin inhibited cell migration and wound healing. This effect could be partially because of suppression of MACC1 expression. However, there have been studies suggesting anti-migratory effects of Rottlerin [209-211, 225], mostly by destabilization of the focal adhesion complex or PKC-δ inhibition followed by reduced E-cadherin expression. These effects need to be verified further and it could be possible that these effects are a consequence of reduced MACC1, thereby contributing to low migratory ability induced by Rottlerin. Similarly for Lovastatin, it has been shown that it reduces migration in colon cancer cells though the mechanism has not been described [226]. Simvastatin, a Lovastatin analogue, was found to inhibit migration in the monocytic cell line THP-1 by decreasing the levels of MMP9 or by inhibiting the production of chemokines [227, 228]. Remarkably, the role of MACC1 in altering levels of MMP9 [127] and in inflammation [147, 148, 229, 230] has already been speculated. Therefore, the decrease in migration via decrease in MMP9 or inhibition of chemokines might be correlated with decrease in MACC1 levels. A detailed study on the mechanism by which MACC1 alters the cell motility and reduce metastatic potential, needs to be investigated in the future.

5.2.3 Both inhibitors interfere with AP-1 and Sp1 mediated transcription of the MACC1 gene

In the first part of our study, we showed that AP-1 and Sp1 play an important role in regulating MACC1 transcription. On identifying Rottlerin and Lovastatin as MACC1 transcriptional inhibitors, we demonstrated the mechanism by which they inhibit MACC1

transcription mediated by AP-1 and Sp1. Treatment of HCT116 cells with both small molecules reduced Sp1 expression which then affected its binding with the MACC1 promoter consequently leading to decreased MACC1 transcription. The role of Rottlerin in inhibiting Sp1 was also previously described in cardiac myofibroblast cells [231]. However, the effect of Lovastatin on Sp1 levels has not been described so far. In this study, analysis of Sp1 mRNA levels revealed that Rottlerin inhibits Sp1 transcription, whereas for Lovastatin, a very small effect was seen at mRNA level but a clear effect was observed at protein level. The difference in the levels of Sp1 mRNA and protein upon treatment with the small molecule inhibitors could be accounted by extensive post translational modifications of Sp1 that regulate Sp1 protein level [232].

In contrast to their effects on Sp1, Rottlerin and Lovastatin treatment increases the level of c-Jun mRNA and protein. However, we demonstrated that c-Jun regulates MACC1 expression and knock-down of c-Jun using siRNA inhibits MACC1 expression. Therefore, this increase in expression levels of c-Jun by MACC1 inhibitors was surprising and suggested that in CRC cells, Rottlerin and Lovastatin control AP-1 mediated MACC1 promoter regulation via a different mechanism. AP-1 is a well-known transcription factor and it translocate into the nucleus and binds to promoters of several genes to activate gene transcription [233]. Using ChIP and EMSA assay, we demonstrated that despite of increased levels of AP-1 upon treatment with Rottlerin and Lovastatin, MACC1 transcription was impaired in CRC cells. Analysis of c-Jun/MACC1 promoter binding revealed that both the small molecule inhibitors disrupt the binding of c-Jun to the MACC1 promoter. This could be either due to inactivation of the DNA binding domain of c-Jun or via inhibition of accessory proteins which are required by Ap-1 to bind to the MACC1 promoter or could be by inhibition of phosphorylation of c-Jun rendering the c-Jun protein ineffective for binding to the MACC1 promoter. However, within the scope of this study it was demonstrated that both the small molecule inhibitors interfere with the transcription factors binding to the MACC1 promoter, thereby impairing MACC1 transcription in CRC cells.

5.3 Rottlerin as a novel treatment option for advanced CRC patients

5.3.1 Rottlerin as a promising MACC1 inhibitor *in vivo*

In this study, both small molecule inhibitors restricted MACC1 expression *in vitro* and show promises as potential MACC1 inhibitors to halt carcinogenesis.

HMG-CoA reductase inhibitors, also called Statins, possess diverse effects beyond their cholesterol-lowering properties and risk of myopathy. Though preclinical data points towards

effectiveness of Statins in demonstrating antineoplastic effects in a variety of tumors, clinical studies have provided conflicting data in the role of statins in prevention or as adjuvant therapy in CRC cancer [234]. There have been lots of speculation and controversy regarding the alarming increase in breast cancer incidence with intake of Statins [235, 236]. However, close inspection of Statin trials is warranted to reveal a specific population at risk with Statin treatment. Further research is needed to assess more precisely the effect and safety profile of statins, or as combination agents with adjuvant or neo-adjuvant chemotherapy. In contrast, Rottlerin *in vivo* potency remains nearly unknown but its promising anti-cancerous effects prospects are greatly anticipated. Thus, we decided to carry out a first *in vivo* study with Rottlerin to assess its role as a MACC1 inhibitor and thus as an inhibitor of CRC progression and provide preclinical data for the justification of clinical studies in humans as a CRC treating approach.

5.3.2 Bioluminescence imaging of xenografted mice revealed anti-tumor potential of Rottlerin

To evaluate *in vivo* efficacy of Rottlerin, we used a metastasis model based on intrasplenic injection of CRC cells into immune-deficient mice. The spleen being intensively blooded provides a way for the transplanted cells to enter the blood circulation [237]. Thus, the intrasplenic model, represent partially the process of metastasis formation and excludes the processes of dissemination from the tumor tissue and invasion into the blood stream [238]. Metastasis formation begins with the local tumor enlargement from which cells invade the vascular and lymphatic system, circulate and finally extravasate into distant organs [239]. So far, no genetic mouse model of spontaneous tumors that arise in the intestines, become invasive, and metastasize to organs such as liver, lungs, and lymph nodes, as they do in humans is available. Thus xenografted models are often used to study the whole progression of the tumor to the metastatic stage. However, the orthotopic model also has several disadvantages, as it is time consuming, complex and only 50% of successful transplanted mice show metastasis to the lymph nodes and very rarely to the liver since majority of the animals die before liver metastasis occur due to tumor burden [238, 240, 241]. Another xenograft intrasplenic model includes implantation of malignant human CRC cells into the spleens of immune-deficient mice. The studies suggest that this model can produce liver metastasis originated from the tumors of the spleen [242]. This animal model is now a popular choice to study primary tumor growth and liver metastasis. Therefore, in this study, we used the intrasplenic mouse model employing endogenously MACC1 expressing

HCT116 cells, to analyze the potential of Rottlerin in halting tumor progression and metastasis caused by MACC1.

Further, tumor progression was monitored using non-invasive bioluminescence imaging to follow the process of MACC1-induced human CRC progression *in vivo*. Bioluminescence imaging is based on the detection of photons, which are excised as a side product in an enzymatic reaction. Bioluminescence imaging in animal models requires a reporter construct that leads to production of the luciferase enzyme. We used HCT116 cells stably expressing a plasmid for the firefly luciferase gene. Firefly luciferase originates from the North American firefly *Photinus Pyralis* and catalyzes the oxidation of luciferin under ATP consumption and emission of light. Monitoring of intrasplenically implanted HCT116 cells into NOD-SCID mice revealed an increased bioluminescence signal in the spleen region over time.

Many non-invasive imaging modalities besides bioluminescence have been described for their use in small animals, such as X-ray computed tomography (CT), magnetic resonance imaging, single photon emission computed tomography (SPECT), positron emission tomography and fluorescence imaging [243]. However, bioluminescence bears several advantages compared to other modalities such as its ease of application, cost effectively and high-sensitivity. It is free from exposure to radiation and provides a relative measure of the signal to the amount of viable cells. Moreover, mammalian tissue has only a very low intrinsic bioluminescence, therefore generates an optimized signal-to-noise ratio [244]. The main limitation of bioluminescence is its anatomic resolution as it only provides 2D images that are relatively low resolution, which is a not very important demand for our study. So we preferred bioluminescence imaging for our study.

The bioluminescence signal from the spleen formed by CRC cells appeared already on day 5 as a weak signal in both solvent and Rottlerin treated mice. In the solvent treated control group, the spleen signal increased overtime until day 27 wherein, the primary tumor became very big and the experiment had to be terminated due to ethical reasons. Rottlerin treatment abrupted the tumor progression by retarding the growth of tumor cells in the spleen and thus reducing the primary tumor size in that organ. Additionally, the level of human MACC1 expression from the mouse spleen bearing human CRC tumor cells was also reduced in the Rottlerin treated group as compared with the control group suggesting that decreased MACC1 levels in the primary tumors contribute to the retarded tumor growth. However, up to day 27, no liver metastasis was observed in solvent treated control group. This was a bit surprising since many comparable studies demonstrated metastasis formation from day 6-8 using the same mouse model [245, 246]. Even the autopsy from the control mice showed clean livers with little or no metastasis. The major limitation with such kind of *in vivo* study is

the determination of the optimal endpoint. The optimal endpoint should be neither too early, otherwise metastases might not be visible, nor too late to reduce the suffering of the animal and avoid tumor burden-caused death. But in this study, the end point came early and animals could not be kept longer to see metastasis in the liver because of large primary tumors in the spleen and had to be sacrificed due to ethical reasons. Hence, the anti-metastatic effect of Rottlerin could not be addressed in this experiment. Nevertheless, the study definitely provides first evidence that Rottlerin inhibits MACC1 expression and impairs the tumor progression of CRC cells *in vivo*. Thus, we conclude that Rottlerin could be exploited as a potential small molecule inhibitor against CRC.

5.3.3 Pros and cons of using small molecule inhibitors for targeting MACC1

In 1800s, bacteriologist Paul Ehrlich coined a term “magic bullet” for chemicals with the ability to specifically target microorganisms. His concept has now emerged in many fields including cancer treatments for therapies other than conventional chemotherapeutic drugs. Such innovative cancer-treatment strategies include development of monoclonal antibodies (mAbs), small molecules, peptide mimetics and antisense oligonucleotides [247] with each class possessing their own share of selectivity, specificity, efficacy and applicability issues.

Using anti-sense oligonucleotides (RNA interference), dose optimization is very complex. Moreover, the systemic application to organisms is challenged by the efficient delivery into the cell [248]. In contrast, both small molecule inhibitors were able to enter the cell without further additives. Systemic Rottlerin application inhibited the MACC1 expression *in vivo* providing the basic evidence for its applicability to treat CRCs. As compared to mAbs and peptide mimetics, small-molecule agents, owing to their small size, can translocate through plasma membranes, therefore be developed to target any molecules regardless of their cellular localization. In addition, therapeutic anti-sense oligonucleotides or mAbs require relatively complex processes with extremely high monetary costs compared with small-molecule inhibitors. In contrast, small-molecule agents are less expensive and have better pharmacological properties [249].

The major issue with small molecule inhibitors remains the specificity. Targeting MACC1 promoter activity with the small molecules will not be solely restricted to the inhibition of MACC1 expression, but might have some off-target effects too. However, in solid cancers, oncogenesis is a multi-step, multi-factorial process and its pathogenesis is not because of a single target. In this context, a single targeted therapy seems theoretically an unfavourable strategy. Therefore, this lack of specificity with small molecules could be potentially

favourable albeit with some risk of increased toxicity which should be assessed during clinical studies.

5.3.4 Summary and Scope: Rottlerin and Lovastatin as potential drugs for treating CRCs

MACC1 was found to be a prognostic biomarker for metastasis-free survival of the CRC patients independent of age, sex, tumor infiltration, nodal status, and lymph vessel invasion. The five-year survival of patients with low MACC1 expression in their primary tumor is 80% which drops to 15% among the patients with high MACC1 level. Therefore, it is now clear that MACC1 plays an important role in CRC progression and metastasis. MACC1 is involved in the crucial step of transition from the benign to the malignant phenotype in the Fearon and Vogelstein carcinoma sequence [135]. Thus, targeting MACC1 holds strong promise in abrupting adenoma to carcinoma progression.

This is the first study identifying small molecule MACC1 inhibitors. Herein, we provide evidence that small molecule drugs, Rottlerin and Lovastatin, inhibit MACC1 expression and thereby MACC1-induced proliferation and cell motility *in vitro*. Both the drugs are transcriptional suppressors which inhibit the binding of c-Jun to the MACC1 promoter and reduce Sp1 levels, collectively leading to decrease MACC1 transcription. Further *in vivo* studies were carried out with Rottlerin alone, and *in vivo* data demonstrated a reduced growth of the primary tumor and inhibition of MACC1 expression in those tumor cells by Rottlerin treatment in xenografted mice. Based on these results, we suggest that Rottlerin might be of clinical relevance in the management of CRC patients. As MACC1 expression in tumors from CRC patients is an indicator for development of metastases [108], Rottlerin is not only able to restrict primary tumor formation but might also inhibit metastasis as well. This should be validated again in a mouse xenograft model that can undergo liver metastasis within an optimal end point time.

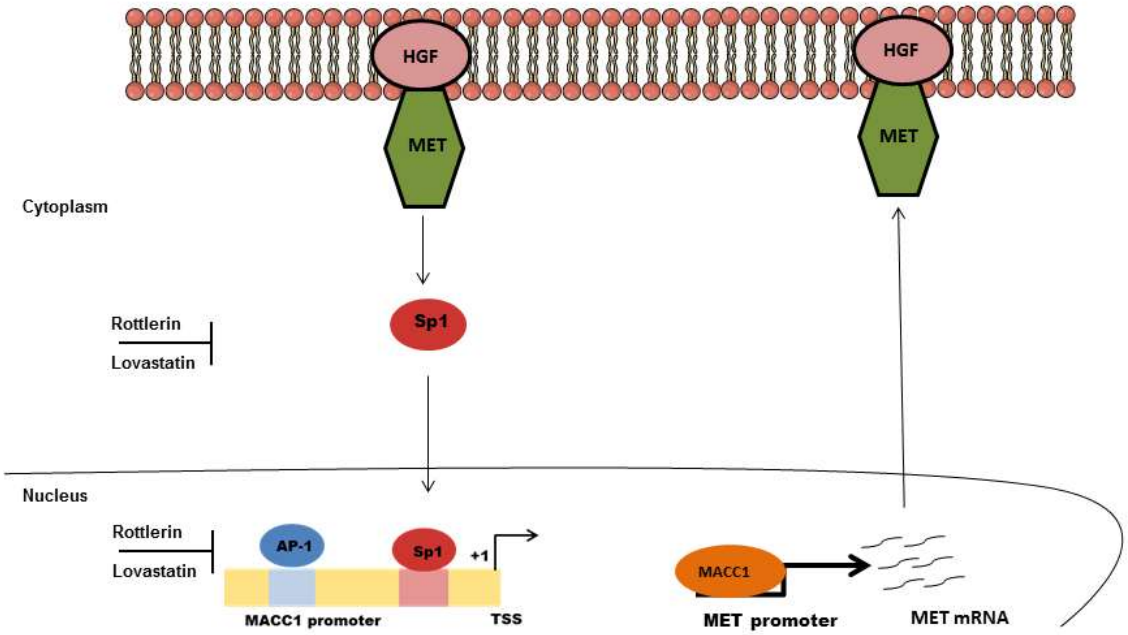


Figure 5.5: MACC1 regulation and its transcriptional inhibition by Rottlerin and Lovastatin. Adapted from Arlt et al. [135]

Moreover, studies performed by Stein and colleagues show, that the level of free circulating MACC1 transcripts in the plasma of CRC patients predict metastases and survival. Thus, patients at high risk of developing metastasis might be monitored based on their MACC1 levels, which can be determined either via immunohistochemistry or qRT-PCR after tumor resection or via blood analysis. For patients with high MACC1 levels, Rottlerin treatment could be of particular value to restrict CRC progression and the development of metachronous metastases.

Current treatment option of CRC patients includes surgical resection along with chemotherapy with drugs, such as 5-Fluoruracil, Leucovorin, Irinotecan or Oxaliplatin which mostly target the increased proliferation rate of cancer cells [250]. In the management of metastatic CRC, targeted therapies such as Cetuximab, Panitumumab or Bevacizumab in combination with above mentioned chemotherapy is used following surgical resection [251]. However, for curing patients with primary CRC that is at high risk for recurrence and metastasis (stage III and high-risk stage II colon cancer), novel drugs targeting the metastatic potential of cells are required, since metastatic dissemination remains the major cause for the mortality associated with CRC. In this respect, Rottlerin presents substantial potential to be tested further into preclinical and clinical studies as neo-adjuvant or adjuvant therapy alone or in combination with standard chemotherapy to target tumor growth as well as to prevent CRC recurrence and metastasis.

6. OUTLOOK

The present study identified the MACC1 promoter and elicited the role of the transcription factors AP-1, Sp1 and C/EBPs in regulating transcription of the MACC1 gene. Current investigations focussed mainly on AP-1 and Sp1 mediated regulation of MACC1 promoter. The possibility of other cis or trans acting transcription factors regulating MACC1 expression still remains and needs to be investigated in the future. To establish the role of GIPC1 as a transcription factor, its nuclear localization studies and further pinpointing the precise position on MACC1 promoter needs to be worked upon by designing more deletion constructs of the MACC1 promoter. In parallel, bioinformatics studies can aid in understanding the region responsible for its shuttling from cytoplasm to nucleus.

The findings of the present study also revealed the potential of Rottlerin and Lovastatin as inhibitors of CRC progression by inhibiting MACC1 transcription. We are currently trying to establishing the role of Rottlerin in not just inhibiting the progression of CRC but also in the inhibition of metastasis associated with the MACC1 expression. Further studies addressing the long term drug tolerability and bioavailability of Rottlerin are required. We propose that in the clinical setting, CRC patients with high MACC1 levels in their primary tumors might be treated with Rottlerin as a targeted therapy to restrict MACC1 associated tumor progression as well as prevent MACC1 induced metastasis and cancer recurrence. Pre-clinical studies with Lovastatin on xenografted mice bearing human CRC cells endogenously expressing MACC1 are currently being performed. For Lovastatin, more preclinical data describing its safety profile is warranted. Moreover, studies including patient stratification on the basis of their previous medical history and genetic background may help in identifying cancer patients who will benefit from statin therapy.

These investigations will further clarify the potential benefits of the clinical application of Rottlerin and Lovastatin on high risk CRC patients in their earlier stages to prevent the progression of the disease to advanced metastatic stage and also to prevent cancer recurrence.

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ERKLÄRUNG

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